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Dual effects of the novel ingenol derivatives on the acute and latent HIV-1 infections

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ABSTRACT

The latent reservoir of HIV-1 in resting memory $CD4^+$ T cells serves as a major barrier to curing HIV-1 infection. Reactivation of latent HIV-1 is proposed as a promising strategy for the clearance of the viral reservoirs. Because of the limitations of current latency reversal agents (LRAs), identification of new LRAs is urgently required. Here, we analyzed Euphorbia kansui extracts and obtained three ingenol derivative compounds named EK-1A, EK-5A and EK-15A. We found that ingenol derivatives can effectively reactivate latent HIV-1 at very low (nanomolar) concentrations in HIV latency model *in vitro*. Furthermore, ingenol derivatives exhibited synergy with other LRAs in reactivating latent HIV-1. We verified that EK-15A can promote latent HIV-1 reactivation in the *ex vivo* resting $CD4^+$ T cells isolated from the peripheral blood of HIV-infected individuals on suppressive antiretroviral therapy. In addition, ingenol derivatives down-regulated the expression of cell surface HIV co-receptors CCR5 and CXCR4, therefore potentially preventing new infection of HIV-1. Our results indicated that the ingenol derivatives extracted from Euphorbia kansui have dual functions: reactivation of latent HIV-1 and inhibition of HIV-1 infection.

1. Introduction

Antiretroviral therapy (ART) can successfully suppresses HIV replication and transforms acquired immune deficiency syndrome (AIDS) into a manageable chronic disease, allowing people infected with human immunodeficiency virus (HIV) to live longer (Gulick et al., 1997; Perelson et al., 1997). However, there remains no cure for HIV infection or AIDS. The major barrier to curing HIV infection is the acquisition of 'provirus' latent reservoirs (Chun et al., 1997; Davey et al., 1999; Finzi et al., 1997; Wong et al., 1997). The HIV latently infected cells can escape antiviral immune response and drugs because they do not generate viral particles while regaining their capacity of virus production when the treatment is interrupted (Peterlin and Trono, 2003). Thus, the focus of research for treatment of HIV has been on purging the latent reservoir in patients (Archin et al., 2014; Deeks et al., 2012). "Shock-and-Kill" (Archin et al., 2012; Deeks, 2012; Rasmussen et al., 2016; Richman et al., 2009) has been proposed to disrupt the latent HIV proviruses by latency reversal agents (LRAs) followed by immune clearance of the virus-infected cells. Several small molecule compounds have been reported to reactivate HIV-1 in latency cell models, including histone deacetylase (HDAC) inhibitors (Archin et al., 2012; Espeseth et al., 2009; Rasmussen et al., 2014; Shirakawa et al., 2013; Wei et al., 2014), the protein kinase C (PKC) agonists (Bedoya et al., 2009; Díaz et al., 2015; Jiang et al., 2014, 2015, 2018; Kulkosky et al., 2001; Miana et al., 2015; Williams et al., 2004; Spivak et al., 2015), histone methyltransferase (HMT) inhibitors (Bernhard et al.,

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Abbreviations

AIDS	acquired immune deficiency syndrome
ART	antiretroviral therapy
BET	bromodomain and extraterminal domain
CCK-8	cell counting kit 8
CDK9	cyclin-dependent protein kinase 9
DMSO	dimethyl sulphoxide
DNMT	DNA methyltransferase
GFP	green fluorescence protein
HDAC	histone deacetylase
HIV	human immunodeficiency virus
HMT	histone methyltransferase
LRA	latency-reversing agents
LTR	long terminal repeat
PBMC	peripheral blood mononuclear cell
PKC	protein kinase C
P-TEFb	positive transcription elongation factor b

2011; Blazkova et al., 2009; Bouchat et al., 2016), the bromodomain and extra terminal domain (BET) inhibitors (Boehm et al., 2013; Li et al., 2013), and other compounds (Doyon et al., 2013; Micheva-Viteva et al., 2011). In recent clinical studies, some of them have been demonstrated to disrupt HIV-1 latency *in vivo*, such as valproic acid (Sagot-Lerolle et al., 2008), vorinostat or SAHA (Archin et al., 2017, 2012), panobinostat (Rasmussen et al., 2014), romidepsin (Wei et al., 2014), bryostatin-1 (Gutiérrez et al., 2016) and disulfiram (Elliott et al., 2015); however, there were limited or no impact on the sizes of latent reservoirs (Delagrèverie et al., 2016; Rasmussen et al., 2013; Rasmussen and Lewin, 2016; Spivak and Planelles, 2016). Developing more potent and safer LRAs is urgently needed.

Natural products derived from microbes and medicinal plants have played an important role in the discovery and development of drugs. Euphorbia kansui has traditionally been used for the treatment of edema, ascites, and asthma (Yan et al., 2014). We previously reported that effective fractions from the dichloromethane extracts of the roots of Euphorbia kansui can reactivate latent HIV-1 replication in different latent cells (The 24th China science technology Forum-High level Forum on HIV cure, December 16–17, 2012, Beijing), and obtained the Chinese patent application approval and authorization (CN102727563B, CN106928063A). More recently, it was reported that ingenol derivatives or crude extracts from Euphorbia kansui could reactivate latent HIV-1 (Cary et al., 2016; Liu et al., 2018) and a clinical trial using Euphorbia kansui extract powder as tea was designed for the purpose of clearing HIV-1 (clinicaltrials.gov, Identifier NCT02531295). In our earlier studies, we found a substantially potent ingenol derivative EK-16A from Euphorbia kansui and proved that it was a PKC agonist that induces both NF-κB and P-TEFb signaling, with a potency in reversing HIV-1 latency (Wang et al., 2017). However, the ingredients of Euphorbia kansuiare varied, and there are more than 12 ingenols along with some other chemical components (Wang et al., 2002).

Here, following our biological screening program on Euphorbia kansui for the discovery of anti-HIV natural products, we have evaluated the components of Euphorbia kansui for their potential in latent HIV-1 reactivation. We obtained three more active ingenol derivatives named EK-1A, EK-5A and EK-15A. Our data indicated that they can effectively reactivate HIV-1 in HIV latently infected cell lines with a low impact on cell proliferation. They can synergize with other LRAs to flush out latent HIV-1 *in vitro*. Also, we verified their efficacies in human primary $CD4^+$ T cells model of HIV latency and in resting $CD4^+$ T cells purified from the peripheral blood of HIV-positive individuals on suppressive ART. Moreover, these compounds down-modulated the expression of HIV cell surface receptors CXCR4 or CCR5, therefore blocking viral spread into uninfected bystander $CD4^+$ T cells. These properties indicate that these ingenol compounds may have potential to develop as new anti-latency candidate drugs for anti-HIV latency studies.

2. Materials and methods

2.1. Sample collection and reagents

Dried root of Euphorbia kansui were collected from Houma, Shanxi province of China. JQ1, 5-Aza, SAHA and PEP005 were purchased from Sigma–Aldrich (Shanghai, China). Prostratin was purchased from LC laboratories (Woburn, MA, USA). Compounds were diluted in anhydrous dimethyl sulfoxide (DMSO) to make 100 mM stock solution and stored at −20 °C.

HIV-1 NL4.3 nano-luc:HIV pseudoviral vector carries nano-luc gene.

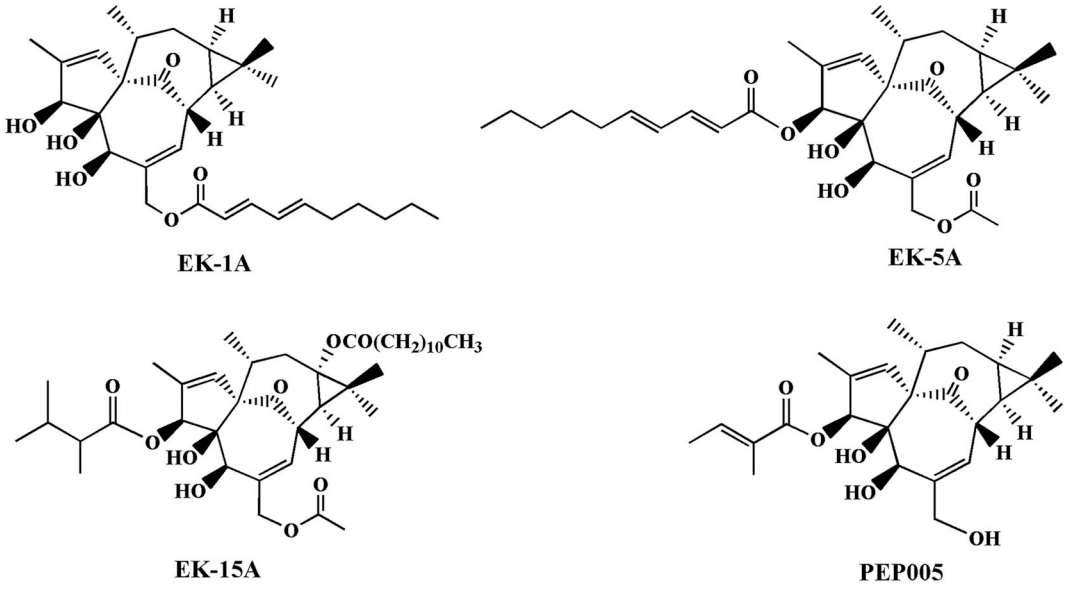

Fig. 1. The chemical structures of ingenol compounds EK-1A, EK-5A, EK-15A and PEP005.

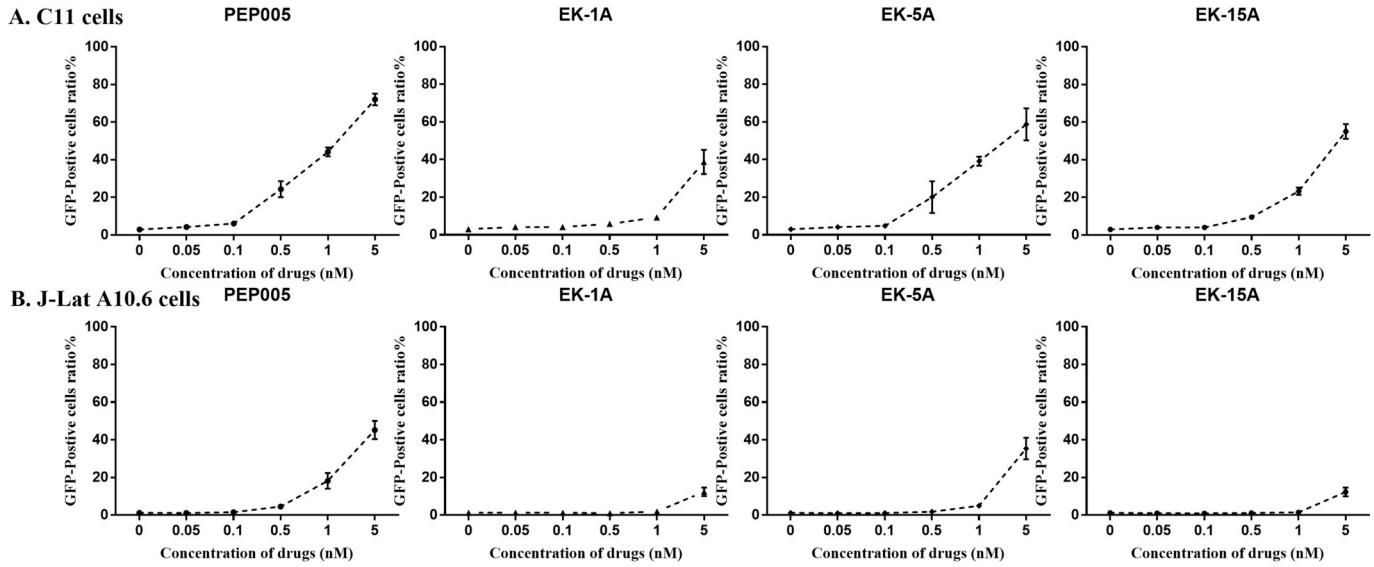

Fig. 2. Dose-dependent effects of EK-1A, EK-5A and EK-15A on reactivation of latent HIV *in vitro*. (**A**) C11 cells and (**B**) J-Lat 10.6 cells were treated with EK-1A, EK-5A, EK-15A or PEP005 at the indicated concentrations. Forty-eight hours post treatment, the percentage of GFP-positive cells was measured by flow cytometry and dose-dependent curves are generated. Data are the means ± s.d. from three independent experiments.

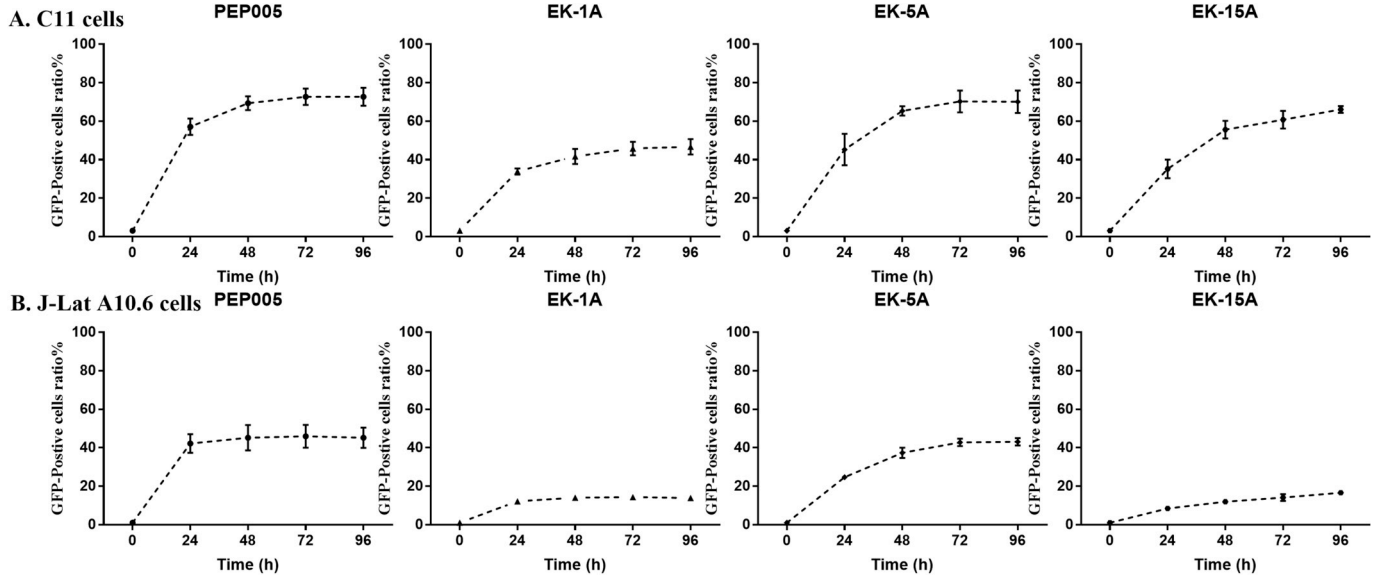

Fig. 3. Time-dependent effects of ingenol compounds EK-1A, EK-5A and EK-15A on the reactivation of latent HIV *in vitro*. (**A**) C11 cells and (**B**) J-Lat 10.6 cells were treated with ingenol compounds EK-1A, EK-5A, EK-15A or PEP005 at 5 nM for up to 96 h. The percentage of GFP-positive cells was measured by flow cytometry and dose-dependent curves were shown. Data show the means ± s.d. from three independent experiments.

The construct was derived from pNL4.3-Luc R-E− (NIH AIDS Reagent Program) and 516bp was double digested by NotI/XhoI restrictive enzymes and introduced then nano-luc gene into the nef gene coding frame of HIV gene.

2.2. Cell lines

C11 and J-Lat 10.6 HIV latency cell lines were used to detect the drug effect of HIV reactivation *in vitro*. C11 HIV latency cell line was established in our lab and had been used before (Ding et al., 2013; Ji et al., 2016; Qu et al., 2013). J-Lat 10.6 cells (Jordan et al., 2003, 2001) were kindly provided by the NIH AIDS Research and Reagent Program. These two cell lines are both HIV latently infected Jurkat cells where GFP expression is a marker for Tat-driven HIV LTR expression.

2.3. Component extraction and isolation of compounds

Dried root of Euphorbia kansui was refluxed with 95% ethanol for 2 h twice. After filtration, the filtrate was evaporated under reduced pressure and the residues were extracted by methylene chloride or petroleum ether and then evaporated to obtain an oily extraction. The extraction was chromatographed on silica gel column using stepwise gradient elution with ethyl acetate and hexane (1:10 to 10:1) followed by HPLC to obtain purified compounds.

2.4. Cell culture

C11, J-Lat 10.6 cells and human peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) at 37 °C under 5% CO_2.

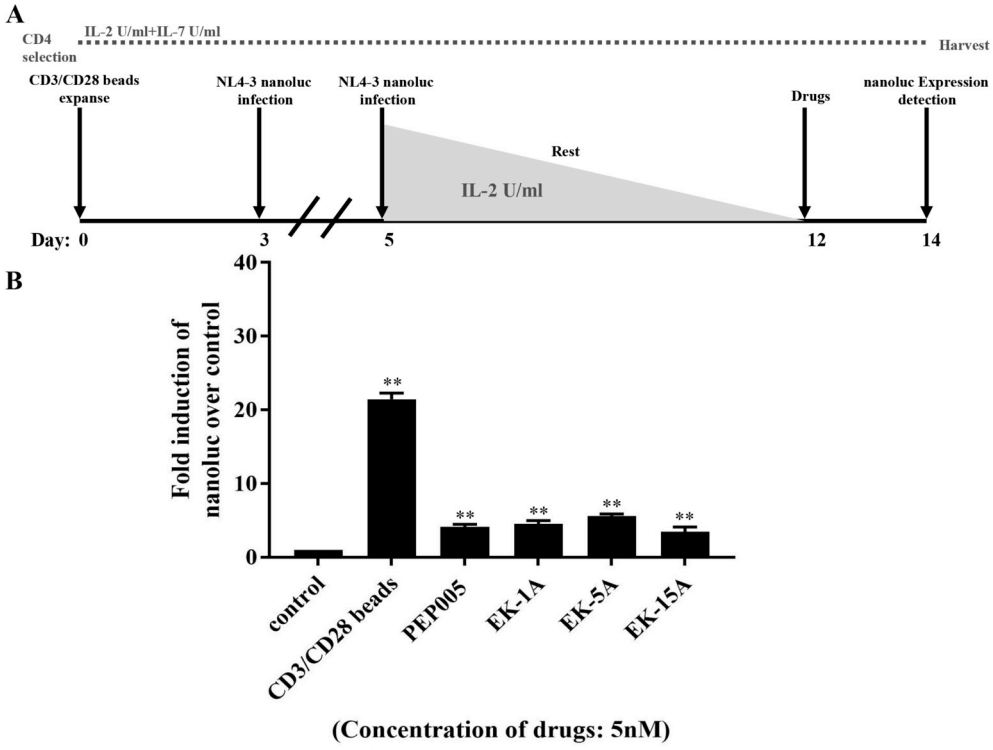

Fig. 4. Ingenol compounds EK-1A, EK-5A and EK-15A reactivate latent HIV-1 in a primary human CD4$^+$ T cell of HIV latency. (**A**) Human primary CD4$^+$ T cells were activated and expanded with CD3/CD28 beads and 30 U/ml IL-2 and IL-7 for 3 days. Cells were infected with HIV-1 NL4.3 nano-luc for 24 h and then infectious virus was removed. After 48 h, cells were infected with the same condition again. After being infected twice, cells were maintained over 7 days with a decreased concentration of IL-2 to establish latency. Uninfected cells were maintained in the same condition for uninfected controls to determine the reactivation of latent HIV. (B) Seven days post infection, cells were stimulated for 48 h with DMSO, CD3/CD28 beads or ingenol compounds PEP005, EK-1A, EK-5A, EK-15A (5 nM), nano-luc expression was measured by Multiscan Spectrum. The fold induction of HIV expression was calculated relative to the control. Data are the means ± s.d. from three independent experiments.

2.5. Flow cytometry

Cells were seeded in a 96-well plate at a density of 2×10^4 cells per well and treated with the obtained compounds, PEP005 (Sigma) or DMSO at the indicated concentrations and different point of time. GFP-positive cells were gated using flow cytometry (Caliber, BD), and data were analyzed with FlowJo Software. The C11 and J-Lat 10.6 cell line both contain an enhanced green fluorescent protein (eGFP) as reporter gene, which is normally expressed at a very low level. When the HIV-1 provirus is reactivated, the protein is expressed at a higher level. We defined the cells expressing the GFP protein as GFP-positive cells. The percentage of GFP-positive cells represents the level of HIV-1 reactivation. All experiments were performed independently at least three times in triplicate per experimental time point.

2.6. In vitro cytotoxicity assay

The Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was used to measure the *in vitro* cytotoxicity of the compounds. Briefly, approximately 4×10^4 cells per well were treated with the obtained compounds or PEP005 for 48 h, and then 10 µl of CCK-8 solution was added to each well of the 96-well culture plates. After 4 h of incubation at 37 °C, the absorbance at 450 nm was measured using a microplate reader.

2.7. Quantitative analysis of latency-reversing agent combinations

We used the Bliss independence model to evaluate the latency-reversing activity of drug combinations. This model is defined by the equation: $fa_{xy,\ P} = fa_x + fa_y - (fa_x)(fa_y)$, where $fa_{xy,\ P}$ is the predicted fraction affected by a combination of drug x and drug y, given the experimentally observed fraction affected by treatment with drug x (fax) or drug y (fay) individually. The experimentally observed fraction affected by a combination of drug x and drug y ($fa_{xy,O}$) can be compared with the predicted fraction affected, which is computed using the Bliss model ($fa_{xy,P}$) as follows: $\Delta fa_{xy} = fa_{xy,O} - fa_{xy,P}$. If $\Delta fa_{xy} > 0$ with statistical significance, then the combined effect of the two drugs exceeds that predicted by the Bliss model and the drug combination displays synergy. If $\Delta fa_{xy} = 0$, then the drug combination follows the Bliss model for independent action. If $\Delta fa_{xy} < 0$ with statistical significance, then the combined effect of the two drugs is less than that predicted by the Bliss model and the drug combination displays antagonism. In our analysis, the fraction affected for the percentage of GFP-positive cells was calculated as follows: fa_x = % GFP-positive cells after treatment with drug x–% GFP-positive cells treated with the DMSO control.

2.8. Isolation of PBMCs and purification of CD4$^+$ T cells

Whole peripheral blood from healthy donors was purchased from the Blood Center of Shanghai (Shanghai, China). The PBMCs isolation was made by the difference of gradient density Ficoll-Hypaque (density = 1.077 g/ml, Haoyang Biological, Tianjin, China). After centrifugation (400 g; 30 min at room temperature), the PBMCs were at the plasma/Ficoll-Hypaque interphase and collected carefully with a Pasteur pipette. After that, the cells were washed twice in PBS (240 g for 10min), and resuspended in RPMI 1640 medium containing 4.5 g/l glucose supplemented with 2 mM L-glutamine, 10%FBS, 100 U/ml penicillin and 100 µg/ml of streptomycin. CD4$^+$ T cells were isolated from PBMCs by negative selection bead sorting (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, PBMC were resuspended in MACS running buffer at 2×10^8 cells/ml and labelled with the appropriate negative selection biotin-antibody cocktail for 10 min at 4–8 °C. Labelled cells were then diluted to 1×10^8 cells/ml in MACS running buffer and incubated with anti-biotin microbeads for an additional 15 min at 4–8 °C. The cells were then washed and resuspended in 500 µl MACS running buffer prior to magnetic cell sorting using an auto MACS (Miltenyi Biotech).

2.9. Human CD4$^+$ T cells model of HIV-1 latency

CD4$^+$ T cells were isolated from PBMCs. After 3-day activation and expansion with CD3/CD28 beads in the presence of 30 U/ml IL-2 and IL-7, the CD3/28 beads were removed from CD4$^+$ T cells culture. Cells were infected with HIV-1 NL4.3 nano-luc for 24 h, then the viruses were

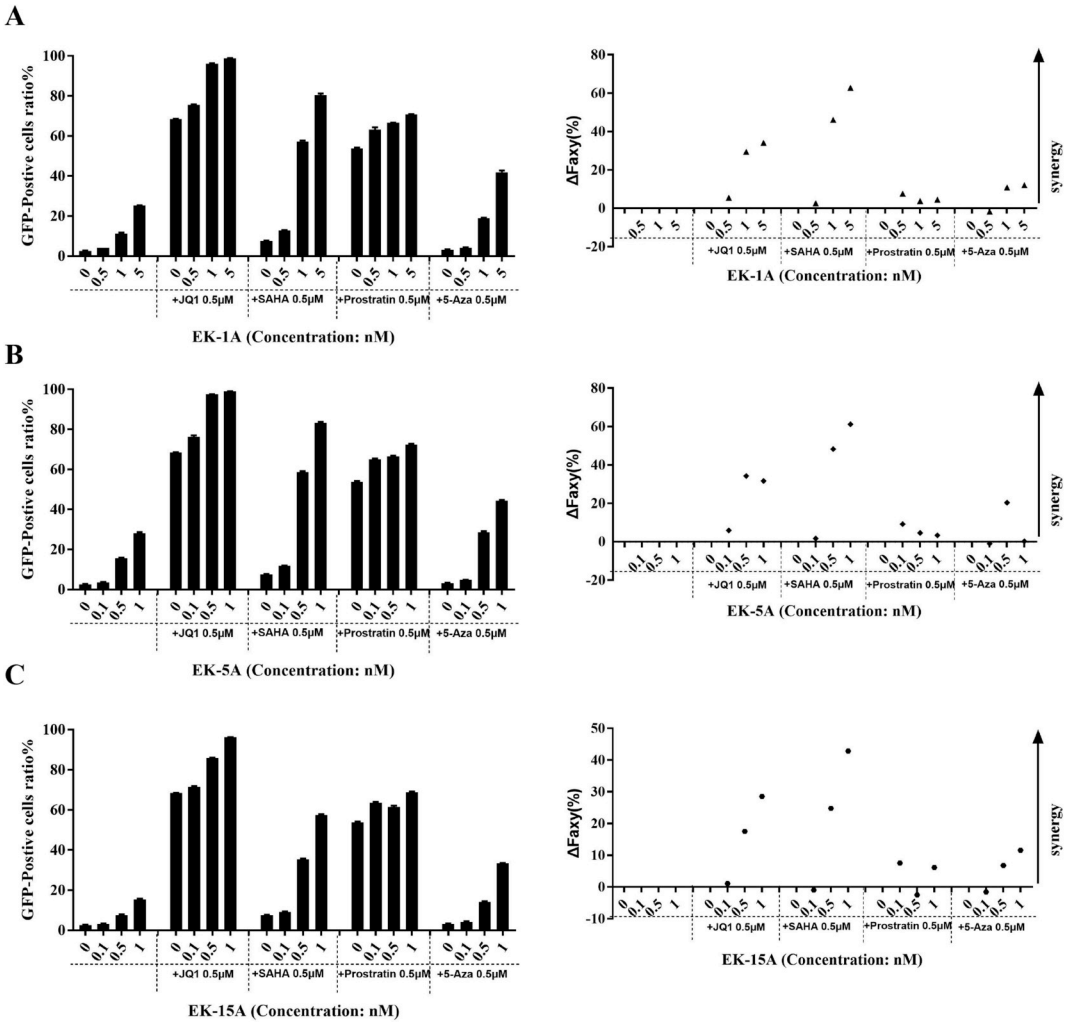

Fig. 5. Ingenol compounds EK-1A, EK-5A and EK-15A synergize with other LRAs in reactivating latent HIV-1 in C11 HIV latent cell model *in vitro*. C11 cells were treated with EK-1A (A), EK-5A (B) and EK-15A (C) alone or in combination with prostratin (0.5 μM), 5-Aza (0.5 μM), JQ1 (0.5 μM), or SAHA (0.5 μM) for 48 h, and the percentage of GFP-positive cells was measured. The Bliss independence model was utilized for calculation of synergy after combination treatment of LRAs (See materials and methods). A solid line signifies pure additive effect (Δfaxy = 0). Synergy is defined as Δfaxy >0 while Δfaxy <0 indicates antagonism. Data are the means ± s.d. from three independent experiments.

removed. Forty-eight hours post infection, cells were infected with the same condition again. After twice viral infection, cells were maintained over 7 days with decreasing concentration of IL-2 to establish latency. Uninfected cells were maintained in the same condition as uninfected controls to determine reactivation. Latently infected cells and uninfected control cells were treated with DMSO, CD3/CD28 beads, PEP005 or ingenols for 48 h, and samples were collected to measure the relative fluorescence unit for latency reversal (Cary et al., 2016; Kim et al., 2014; Pandeló José et al., 2014; White et al., 2016).

2.10. Detection of T cell markers

The primary $CD4^+$ T cells isolated from healthy individuals were incubated with ingenols in 96-well plates for 48 h and immunostained with anti-CD25, anti-CD69, anti-CD4, anti-CCR5 or anti-CXCR4 antibodies (BD-Biosciences) for 20 min at 4 °C. Cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry.

2.11. Isolation of resting $CD4^+$ T cells

Four HIV-1-infected individuals receiving suppressive ART were recruited by the Shanghai Public Health Clinical Center for this study. All research participants in this study gave written informed consent. All individuals had received ART for at least 6 months and maintained blood plasma HIV-1 RNA to undetectable levels (lower than 50 copies/mL) at the time of the study. $CD4^+$ T lymphocytes were purified as described before. And then the resting $CD4^+$ T lymphocytes were further enriched by depletion of $CD69^+$, $CD25^+$ or human leukocyte antigen DR+ (HLA-DR+) cells (Bullen et al., 2014).

2.12. Measurement of cell associated HIV-1 RNA transcripts

Purified resting $CD4^+$ T cells (1×10^6) were treated with EK-15A or EK-16A for 18 h in the presence of 10 μM T20 and collected for RNA purification. Total RNA was extracted using an RNeasyPlus Mini Kit (QIAGEN) following the manufacturer's protocol, and cDNA synthesis was performed using a QuantiTect Reverse Transcription Kit (QIAGEN). Real-time PCR was performed in triplicate using the QuantiFast SYBR Green PCR Kit (QIAGEN) on a Roche LightCycler 480 II machine. Primers and probes used for detecting the intracellular RNA were: forward (5′–3′) CAGATGCTGCATATAAGCAGCTG (9501–9523), reverse (5′–3′) TTTTTTTTTTTTTTTTTTTTTTTTGAAGCAC (9629-polyA) and probe (5′–3′) FAM-CCTGTACTGGGTCTCTCTGG-MGB (9531–9550). Data from the triplicate samples were averaged and presented as fold change relative to DMSO control (Shan et al., 2013).

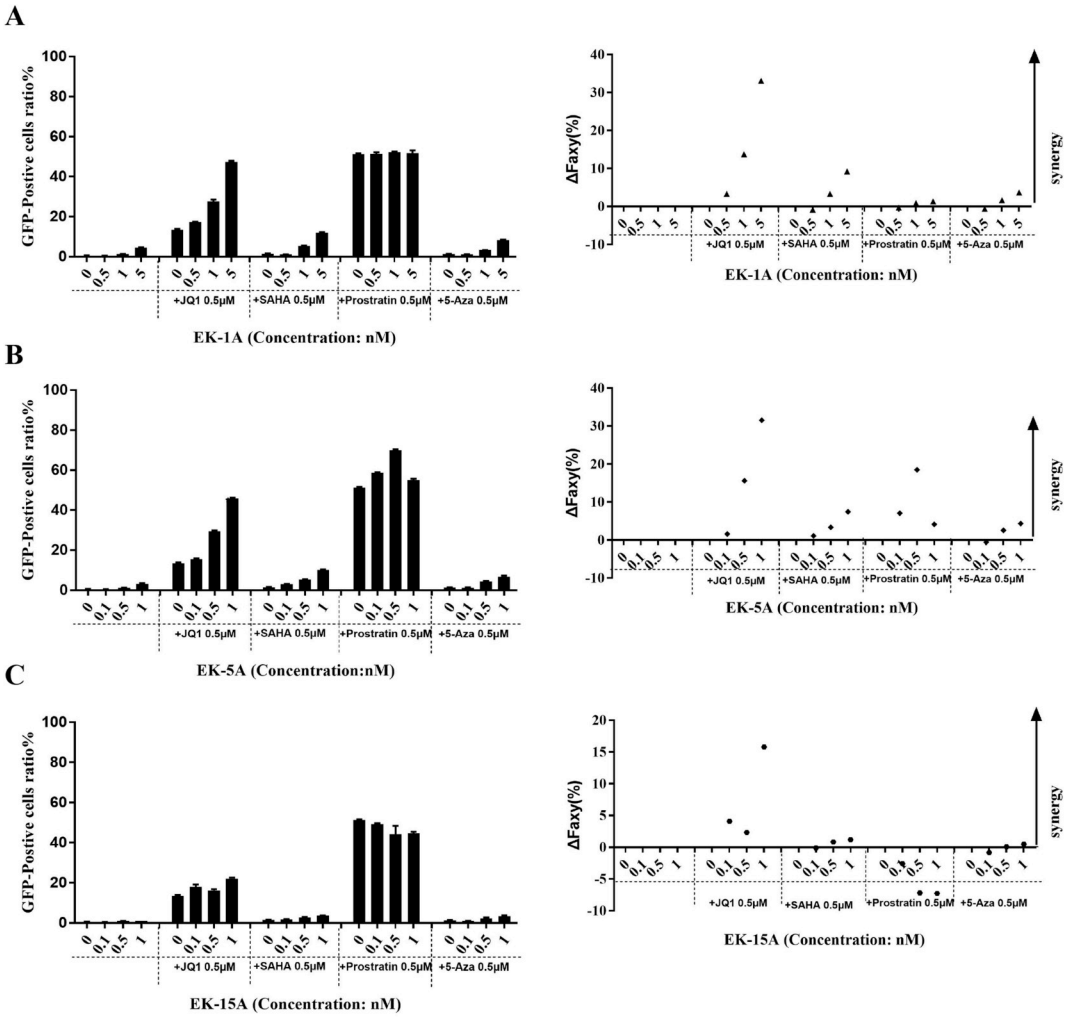

Fig. 6. Ingenol compounds EK-1A, EK-5A and EK-15A synergize with other LRAs in reactivating latent HIV-1 in J-Lat A10.6 cells *in vitro*. J-Lat A10.6 Cells were treated with EK-1A (A), EK-5A (B) and EK-15A (C) alone or in combination with prostratin (0.5 μM), 5-Aza (0.5 μM), JQ1 (0.5 μM), or SAHA (0.5 μM) for 48 h, and the percentage of GFP-positive cells was measured. The Bliss independence model was utilized for calculation of synergy after combination of LRA treatment (See materials and methods). A solid line signifies pure additive effect (Δfaxy = 0). Synergy is defined as Δfaxy >0 while Δfaxy <0 indicates antagonism. Data show the means ± s.d. from three independent experiments.

2.13. Virus infection of primary CD4$^+$ T cells and detection of antigen p24 levels by ELISA

Primary CD4$^+$ T cells isolated from blood samples from healthy donors were pre-treated with EK-15A or EK-16A for 48 h. Then, the CD4$^+$ T cells were infected with replication-competent HIV-1 virus from patient cell supernatant through spinoculation. Following an exposure of 24 h to HIV-1, the cells were washed off and cultured in the fresh medium. After 72-h infection, cell supernatants were collected and HIV-1 p24 levels were measured using the HIV-1 p24 Antigen ELISA Kit (R&D System, Minnesota, USA) according to the manufacturer's instructions.

2.14. Statistical analysis

Data are representative of three independent experiments, and error bars represent ±SD. Two treatment groups were compared by two-tailed unpaired Student t-test, using Microsoft Excel and Prism 7.0. Statistical significance was indicated as *$p < 0.05$, **$p < 0.01$.

3. Results

3.1. EK-1A, EK-5A and EK-15A isolated from Euphorbia kansui can reactivate latent HIV-1

We have obtained three HIV-1 latency-reversing products derived from our previously reported but unpurified Euphorbia kansui by column chromatography and HPLC (Wang et al., 2017). These three newly identified ingenols are: 20-O-(2′E,4′E-decadienoyl) ingenol called EK-1A, 3-O-(2′E,4′Z-decadienoyl)-20-O-acetylingenol called EK-5A, and 3-O-(2,3-dimethylbutanoyl)-13-O-dodecanoyl-20-O-acetylingenol called EK-15A. Their chemical structures were shown in Fig. 1. EK-1A, EK-5A and EK-15A are structurally analogous to PEP005 (Fidler et al., 2014), a previously FDA-approved drug for the treatment of precancerous actinic keratosis, which was reported to effectively reactivate latent HIV-1 *in vitro*. Therefore, we used it as a positive control in this study. To test their ability in latency, two HIV-1 latency cell models, C11 cells and the J-Lat 10.6 cells, were treated with different concentrations of ingenols (EK-1A, EK-5A and EK-15A) and PEP005. The GFP expression was measured by fluorescence microscopy and flow cytometry 48 h after drug treatment. In

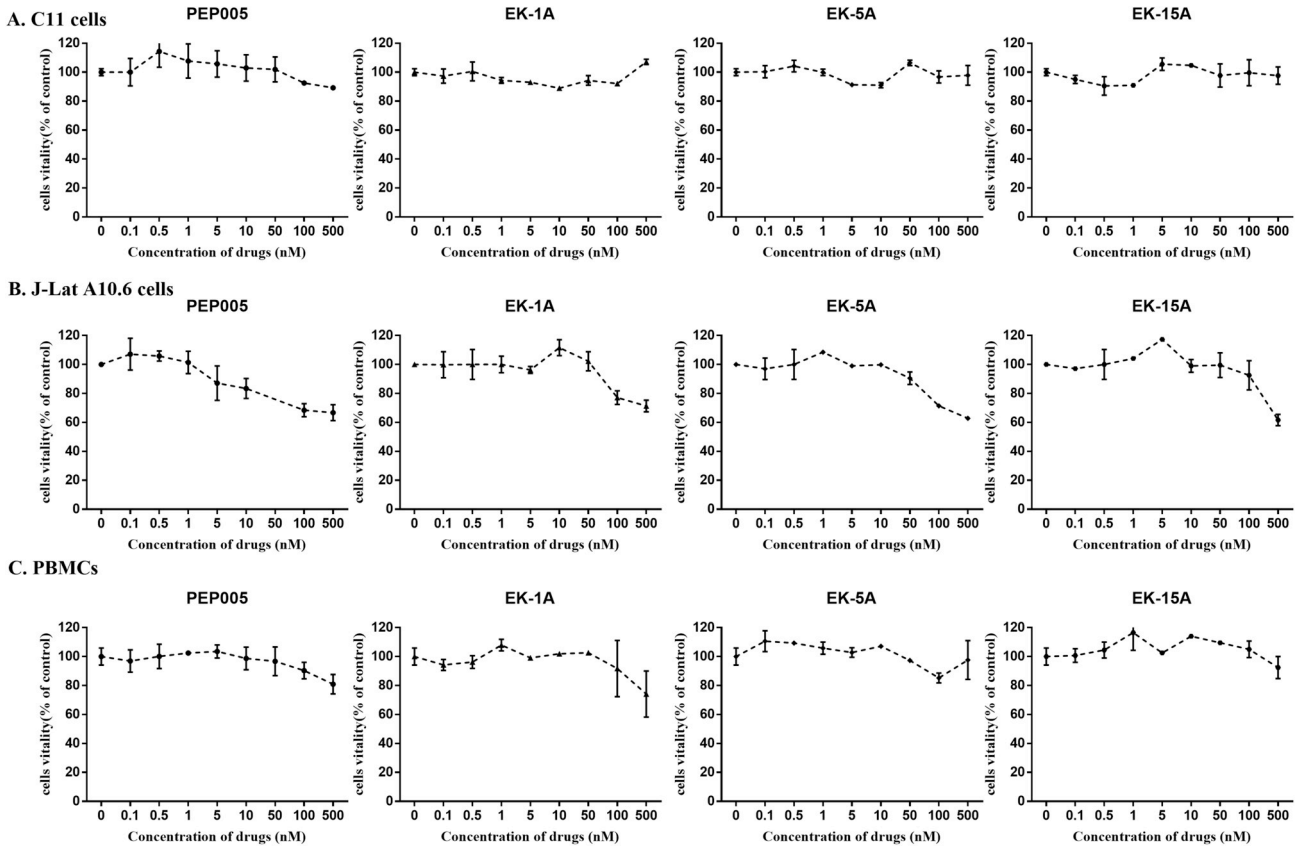

Fig. 7. Effects of ingenol compounds EK-1A, EK-5A and EK-15A on cell viability. C11 cells (**A**), J-Lat 10.6 cells (**B**) and PBMCs (**C**) were treated with EK-1A, EK-5A, EK-15A or PEP005 for 48 h, then the cell viability was measured by CCK-8 kit (Dojindo). The division of OD450 between treated and control groups indicate the percentage of cell viability. Data are the means ± s.d. from three independent experiments.

C11 cells, HIV LTR-driven GFP expression was enhanced from about 3% to 70% with the concentration ranged between 0.05 nM and 5 nM (Fig. 2A, Supplementary Figure 1). It was also found that GFP expression was increased from about 3% to 73% in 5 nM in time-dependent manner. After 48-h treatment, the expression of GFP tended stable (Fig. 3A, Supplementary Figure 3). In the J-Lat 10.6 cells, a similar trend was observed at different concentrations and time points after ingenol treatment. GFP-positive cells were increased up to about 40% in a concentration dependent manner from 0.05 nM to 5 nM (Fig. 2B, Supplementary Figure 2). At the concentration of 5 nM, GFP-positive cells were induced up to about 40% and then remained stable (Fig. 3B, Supplementary Figure 4). Taken together, these data showed that ingenols EK-1A, EK-5A and EK-15A stimulated latent HIV-1 reactivation in a dose- and time-dependent manners in C11 and J-Lat 10.6 cell line of HIV latency.

3.2. Ingenols EK-1A, EK-5A and EK-15A reactivate HIV-1 in a human CD4$^+$ T cell model of HIV-1 latency

Resting CD4$^+$ T lymphocytes are the body reservoir of transcriptionally silent HIV in infected individuals; hence, these cells are among the best models of HIV latency to evaluate the latency reversal (Cary et al., 2016; Kim et al., 2014; Pandeló José et al., 2014; White et al., 2016). The CD4$^+$ T cells were isolated from healthy human peripheral blood and spinoculated with HIV-1 NL4.3 nano-luc. The cultured was stimulated with reduced IL-2 concentrations to induce cell quiescence and HIV-1 latency, and test the nano-luc expression to confirm that our culture conditions induced a latent state. The HIV latently infected cells were then reactivated with DMSO (control), CD3/CD28 beads or ingenols at 5 nM for 48 h and samples were collected to measure the relative fluorescence unit (Fig. 4A). After 48-h treatment, PEP005, EK-1A, EK-5A and EK-15A induced a 4-, 5- and 3-fold increase of nano-luc expression (Fig. 4B), confirming that EK-1A, EK-5A and EK-15A induced a latency reversing.

3.3. Synergistic activation of HIV-1 by EK-1A, EK-5A and EK-15A in combination with other LRAs

To test if EK-1A, EK-5A and EK-15A can cause synergistic reactivation of latent HIV when combined with other LRAs, PKC agonist prostratin, DNA methyltransferase (DNMT) inhibitor 5-azacytidine (5-Aza), BET inhibitors JQ1 and HDAC inhibitor vorinostat (SAHA) were co-administered with ingenols in latent HIV-infected cell line C11 for 48 h and assayed by flow cytometry. As shown in Fig. 5, EK-1A, EK-5A and EK-15A all demonstrated synergy with JQ1, SAHA, 5-Aza or prostatin. When tested in J-Lat 10.6 cells (Fig. 6), EK-1A demonstrated synergy with JQ1, SAHA, 5-Aza but had no synergy with prostatin. EK-5A showed synergy with all four agents JQ1, SAHA, prostratin or 5-Aza. EK-15A demonstrated a synergy with JQ1 or SAHA, but had no synergistic effect in combination with prostatin or 5-Aza. Taken together, these data indicate that EK-1A, EK-5A and EK-15A synergizes with 5-Aza, JQ1, SAHA or prostatin to reactivate the latent HIV-1 in the Jurkat T cell line model of HIV latency.

3.4. Ingenols EK-1A, EK-5A and EK-15A have little cellular toxicity in T cells

To determine whether EK-1A, EK-5A and EK-15A affect cell growth, we measured the cell viability of Jurkat cell lines and the PBMCs from healthy donors after treating the cells with EK-1A, EK-5A, EK-15A and PEP005 for 48 h. As indicated in Fig. 7, EK-1A, EK-5A and EK-15A had minimal effects on cell growth. There was no significant reduction in the cellular viability in up to 500 nM of these compounds, indicating

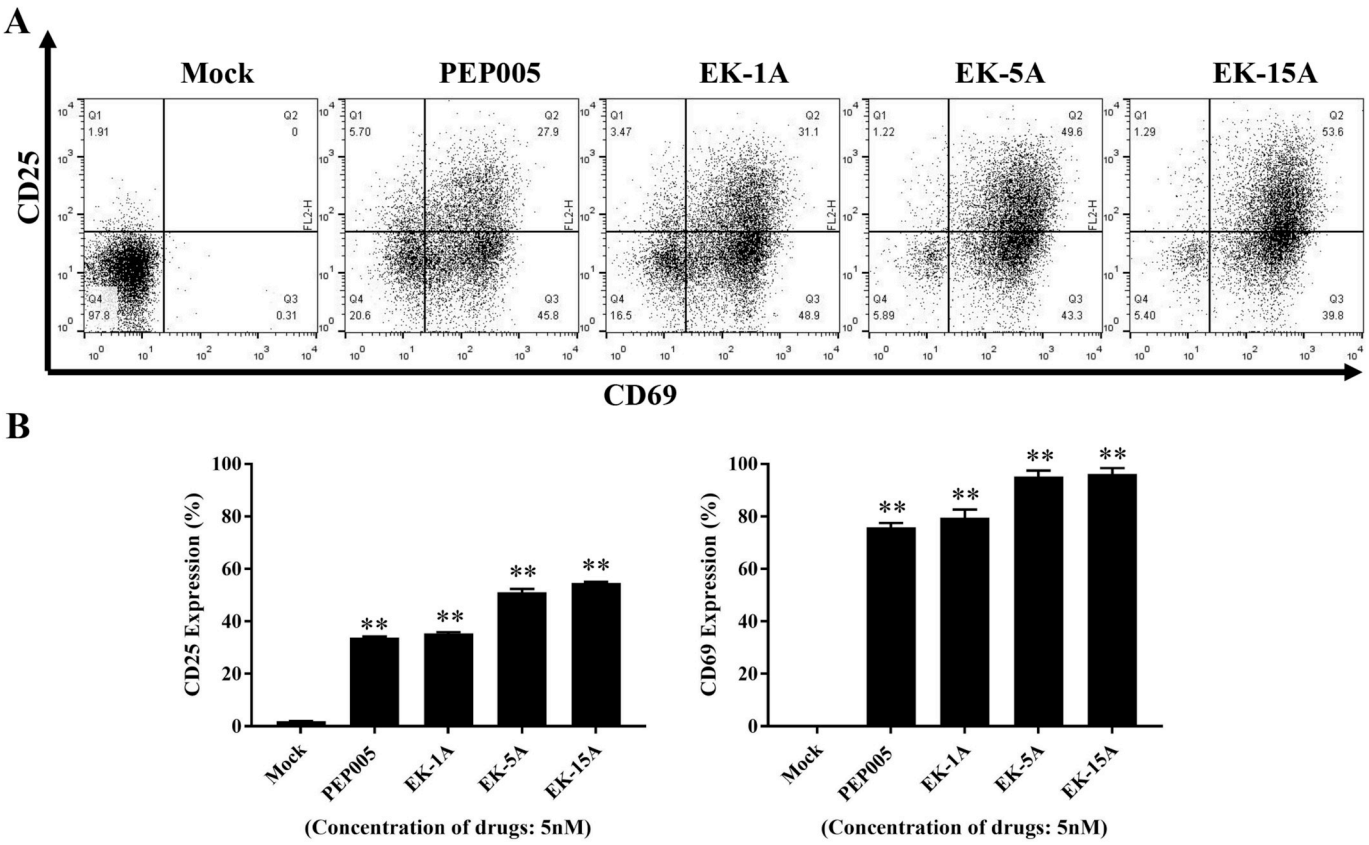

Fig. 8. The effect of ingenol compounds EK-1A, EK-5A and EK-15A on the expression of CD25 and CD69. (**A**) Human $CD4^+$ T cells were treated with either EK-1A, EK-5A, EK-15A or PEP005 (5 nM) for 48 h and the expression of CD25 and CD69 was detected by flow cytometry using antibodies against CD25 and CD69. (**B**) Summary of effects of ingenols EK-1A, EK-5A, EK-15A or PEP005 (5 nM) on CD25 and CD69 expression were presented as histograms. *$p < 0.05$, **$p < 0.01$, compared with control treatment.

that they are safe at its active concentrations in reactivation of latent HIV-1.

As reported before, all known ingenols used in reversal of HIV-1 latency seem to have a similar induction effect of immune activation. Some scientists considered that some degree of T cell activation may be required for an efficient viral reactivation to occur (Jiang et al., 2014; Pandeló José et al., 2014; Spivak et al., 2018; Spivak and Planelles, 2017). We examined the expression of T cell activation biomarkers using primary $CD4^+$ T cells purified from PBMCs of healthy HIV-negative donors following 48 h of stimulation with EK-1A, EK-5A, EK-15A and PEP005. Similar to PEP005 (Fig. 8), treatment with EK-1A, EK-5A, or EK-15A was associated with increased expression of CD69, an early marker for T lymphocytes activation. This is similar to the results reported by Jiang et al., that PEP005 caused increasing CD69 expression in $CD4^+$ T cells. EK-1A, EK-5A, EK-15A and PEP005 all have some effects on the expression of CD25, but to a much less extent.

3.5. Ingenols EK-1A, EK-5A and EK-15A down-modulate CXCR4 and CCR5 expression in $CD4^+$ T cells

Expression of HIV-1 receptors/co-receptors is important for viral attachment and entry into immune cells. To investigate how ingenols influence the expression of virus-specific surface receptors, human $CD4^+$ T cells were treated with EK-1A, EK-5A and EK-15A at 5 nM, followed by an evaluation of the expression levels of CD4, CCR5, and CCXR4 by using flow cytometry. Our data showed that EK-1A, EK-5A and EK-15A caused a significant reduction in the expression of CCR5 and CXCR4 (Fig. 9), suggesting that EK-1A, EK-5A and EK-15A may not pose the risk of increasing susceptibility of $CD4^+$ T cells to HIV-1 infection during its reactivation of HIV latency. Instead, they may help prevent HIV-1 infection of naïve $CD4^+$ T cells. These findings suggest that EK-1A, EK-5A and EK-15A can down-modulate HIV-1 co-receptor expression (CXCR4 and CCR5) that may be beneficial during the process of latent HIV-1 reactivation to prevent HIV infection of naïve $CD4^+$ T cells.

3.6. EK-15A induces HIV-1 expression in the resting $CD4^+$ T cells from ART–treated patients

Considering their comprehensive effects during acute or latent HIV infection, we chose EK-15A to investigate its antiviral effect *ex vivo*. Purified resting $CD4^+$ T cells from four patients under suppressive ART were treated with 5 nM EK-15A or EK-16A, and cellular HIV-1 reactivation was examined by HIV-1 mRNA expression using qRT-PCR with primers/probe targeting HIV-1 3′ polyadenylation (poly A) region. The results showed that EK-15A treatment induced full length HIV-1 transcripts in cells from all 4 donors, with a mean induction of about 1.70-fold (Fig. 10), and EK-16A shows a mean induction of about 1.81-fold. These results suggest that EK-15A could reactivate transcription of HIV-1 from the resting $CD4^+$ T cells of ART-suppressed patients *ex vivo*.

3.7. EK-15A inhibits HIV-1 infection of primary $CD4^+$ T cells ex vivo

Our data showed that these three novel ingenols caused a reduction in the expression of HIV-1 co-receptors, suggesting that they may contribute to suppression of propagating HIV-1 infection of $CD4^+$ T cells following the reactivation of latent HIV-1. To test this idea, the primary $CD4^+$ T cells were infected with HIV-1 with or without pre-treatment of EK-15A/EK-16A for 48 h, then the $CD4^+$ T cells were continued to incubate without EK-15A for 72 h, supernatants were then collected for p24 ELISA. We

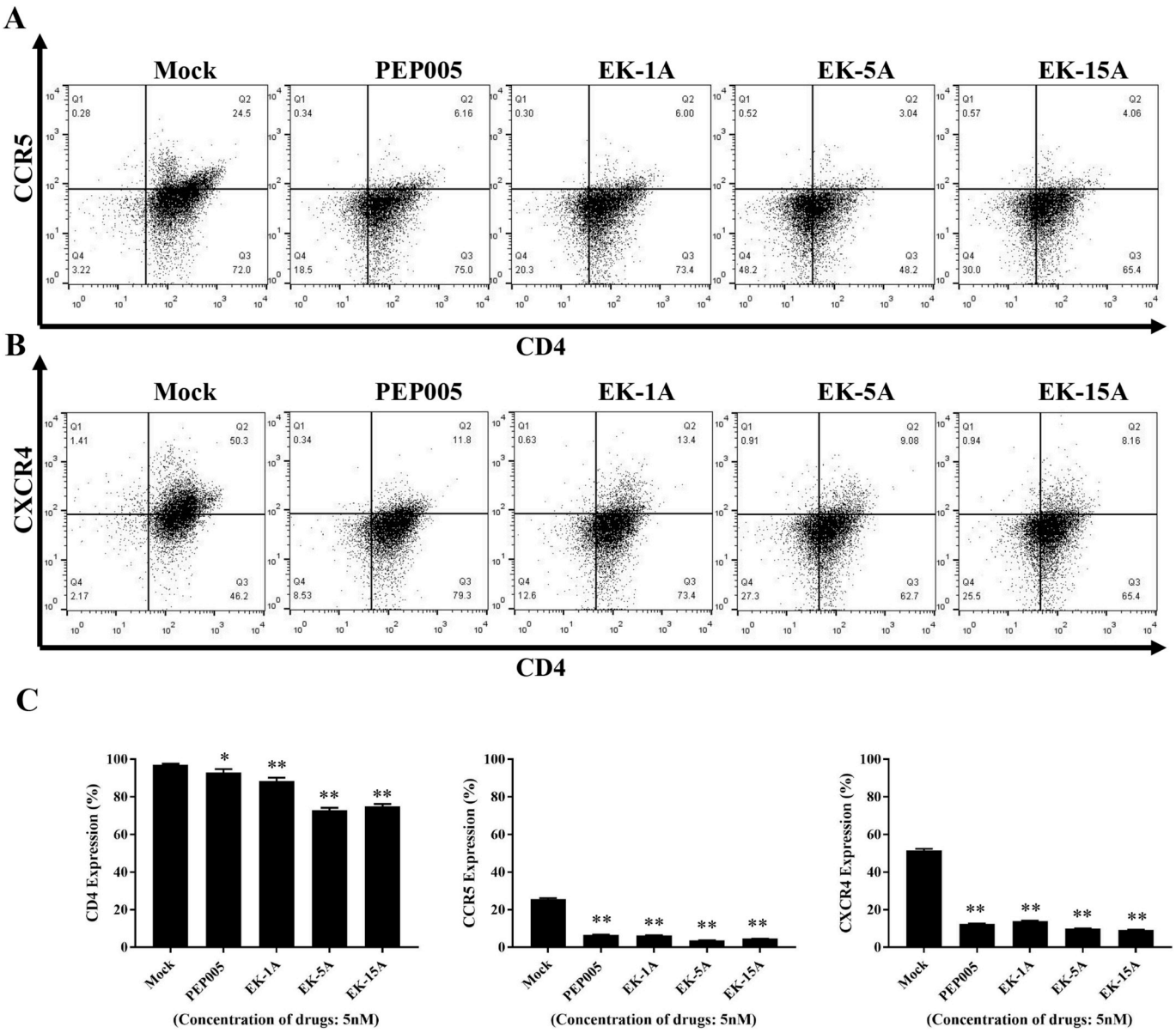

Fig. 9. The effect of ingenol compounds EK-1A, EK-5A and EK-15A on the expression of CD4, CCR5 and CXCR4. (**A** and **B**) Human primary $CD4^+$ T cells were treated with either EK-1A, EK-5A, EK-15A or PEP005 for 48 h, and the expression of CD4, CCR5 and CXCR4 was detected by flow cytometry using antibodies against CD4, CCR5 and CXCR4. (**C**) Summary of effects of EK-1A, EK-5A, EK-15A or PEP005 on CD4, CCR5 and CXCR4 expression are presented as histograms. *$p < 0.05$, **$p < 0.01$, compared with control treatment.

observed that EK-15A decreased the level of viral replication of primary $CD4^+$ T cells (Fig. 11). These observations confirmed that this novel ingenol does prevent new infection of bystander $CD4^+$ T cells.

4. Discussion

Compounds derived from plant, marine and other natural products can target HIV latent reservoirs. Diterpenes from latex or radix of Euphorbia lacteal (Avila et al., 2010), Euphorbia tirucalli (Abreu et al., 2014), Euphorbia umbellate (Valadao et al., 2018) and Euphorbia kansui (Cary et al., 2016; Liu et al., 2018) have been studied to reactivate HIV-1 provirus *in vitro*. Using HIV-1 latently infected C11 cells, we screened extracts from Euphorbia kansui to discover new compounds that can antagonize HIV-1 latency for further investigation.

In our earlier studies, we found a substantially potent ingenol derivative EK-16A isolated from Euphorbia kansui and proved that it is a PKC agonist that induces both NF-κB and P-TEFb signaling, with a potency in reversing HIV-1 latency (Wang et al., 2017). Here, we report three more novel ingenol diterpenes, EK-1A, EK-5A and EK-15A, from Euphorbia kansui. We found that EK-1A, EK-5A and EK-15A can significantly reactivate HIV-1 from latency at a nanomolar level of concentration in HIV latently infected Jurkat cell lines and primary $CD4^+$ T cell model of latency. Especially, EK-15A promoted HIV-1 reactivation in the resting $CD4^+$ T cells purified from patients on suppressive ART *ex vivo*. As Euphorbia plants are widely distributed and rich in number of ingenols, these compounds can be obtained easily from this plant than chemical synthesis. They have the potential to be candidate compounds to interrupt HIV-1 latency. Previous studies suggest that the efficiency in upregulating the HIV LTR varies and depends on the nature and position of the esters in the diterpenes ring, such as modification of side chain at carbons 3 and 13 (Blanco-Molina et al., 2001; Kulkosky et al., 2004; Liu et al., 2018; Pandeló José et al., 2014; Jiang et al., 2015). Studies with these new ingenols suggest that changes in carbon 20 also affect the potential of reactivation, indicating that investigation into

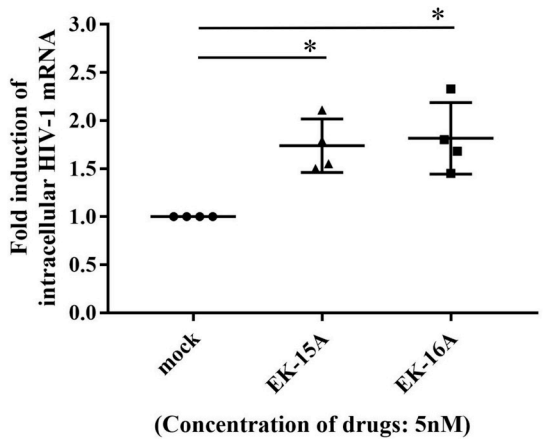

Fig. 10. Ingenol compound EK-15A induces the reactivation of latent HIV-1 in resting CD4$^+$ T cells isolated from cART-suppressed patients. Primary CD4$^+$ T cells were isolated from the peripheral blood of HIV positive individuals on suppressive ART, then the cells were treated with 5 nM of EK-15A or EK-16A for 48 h. Cell associated HIV-1 mRNA levels was quantified by RT-qPCR and presented as fold induction relative to mock-treated control. *p < 0.05, **p < 0.01.

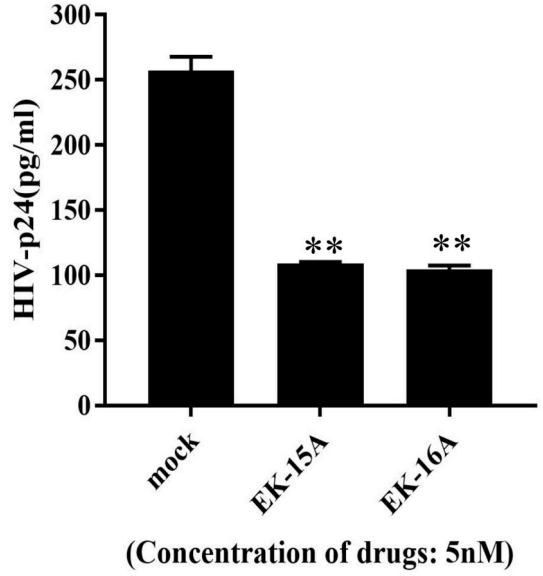

Fig. 11. Ingenol compound EK-15A inhibits HIV-1 infection of primary CD4$^+$ T cells ex vivo. Primary CD4$^+$ T cells were infected with HIV-1 with or without pre-treatment of 5 nM EK-15A or EK-16A for 48 h, then the compounds were washed out and continued to culture for 72 h. Supernatants were collected for the detection of p24 by ELISA assay. *p < 0.05, **p < 0.01.

structure modification of ingenol side chains may identify novel ingenols with unique biological function in reactivation of latent HIV so that ingenols with high activity in latency reversal but with lower cytotoxicity and immune activation could be discovered. Synergistic reactivation of latent HIV-1 was also achieved by combining these three compounds with 5-Aza, JQ1, SAHA or prostratin, this is similar to other inegnols (Jiang et al., 2015; Darcis et al., 2015). Ingenols have complicated chemical structures which are hard to chemically synthesize although a 14-step of chemical synthesis of ingenol has been published before (Jørgensen et al., 2013). Our ingenol compounds can be relatively easier to isolate from a massive scale cultivation of Chinese Kansui. Furthermore, although ingenols such as IngB, PEP005, and Ingenol-3,20-dibenzoate have been extensively tested *in vitro*, *ex vivo* and/or *in vivo*, none of them has been systematically administrated in patients (Jiang et al., 2014, 2015, 2018; Spivak et al., 2015). Chinese Kansui has been safely used in Chinese population as a medicine for the treatment of immune diseases for thousands of years, indicating that our newly discovered ingenols may have potentials to advance into *in vivo* study in the future. Although individually, they are not superior to PEP005 in reactivation of latent HIV, it may be worth testing them in combination together but with a much lower individual dosage. This warrants *in vitro* and *in vivo* investigations in the future.

To be clinically applicable, effective LRAs should be not only highly potent but also display low cytotoxic to immune cells (Jiang and Dandekar, 2015). Ingenol compounds EK-1A, EK-5A and EK-15A showed relatively low cellular toxicity on Jurkat cell lines and human PBMCs after prolonged exposure of 48 h, indicating that these compounds may not cause significant impacts on cell proliferation. However, there was an indication that EK-1A, EK-5A and EK-15A up-regulated high level of CD69 and some level of CD25 expression, which is similarly found in other PKC agonists. This reminds us again that combination therapy by targeting different signaling pathways could be an ideal tool to combat with HIV latency. Or, like the concept of traditional Chinese medicine, it might be wise to test a combination of our newly defined three ingenols in reactivation of latent HIV but with a lower level of individual ingenol. It should be noted that some degree of immune activation may be required for an efficient reactivation of latent HIV (Jiang and Dandekar, 2015) which may not necessarily lead to the global T-cell activation (Blázquez et al., 2002). In addition, Planelles' group recently proposed that in combination of ingenol compound with JAK inhibition not only it can maintain the potency of ingenols to flush out latent HIV but also reduce PKC agonist-induced immune activation (Spivak and Planelles, 2016). Since the expression of CD69 is dependent on NF-κB binding to its promoter region, these findings provide evidence that EK-1A, EK-5A and EK-15A reverse HIV-1 latency may act through PKC/NF-κB signaling to reactivate latent HIV.

The ability of EK-1A, EK-5A and EK-15A to decrease expression of T cell surface markers CCR5 and CXCR4 directly impacts the susceptibility of CD4$^+$ T cells to HIV-1 infection *ex vivo*. Our findings suggest that EK-15A helps prevent HIV infection of CD4$^+$ T cells through down-modulation of HIV co-receptor expression, which is similar as EK-16A as we reported before. This activity may be beneficial during the latency reversal in patients.

In summary, ingenol derivatives EK-1A, EK-5A and EK-15A can reactivate latent HIV-1 and more likely have the ability to block *de novo* HIV-1 infection, which make them the potential candidates for advancing into preclinical HIV cure studies. Our study along with previous reports suggests that ingenol derivatives, including EK-1A, EK-5A and EK-15A, represent a new group of leading compounds for combating HIV latency.

Conflicts of interest

The authors declare no competing financial interests.

Contribution

HZ. conceived and designed the experiments. HY and XL carried out most experiments. XY, PL, YW, ZJ, HP, LZ, YZ, YS, HL and TZ participated in some of the experiments. HZ, HW and ZM directed and supervised the experiments and interpretation of data. HY, IK, HZ and GJ wrote the main manuscript text. All authors reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104555.

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