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4-deoxyphorbol inhibits HIV-1 infection in synergism with antiretroviral drugs and reactivates viral reservoirs through PKC/MEK activation synergizing with vorinostat

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1 **ABSTRACT**

2 Latent HIV reservoirs are the main obstacle to eradicate HIV infection. One strategy
3 proposed to eliminate these viral reservoirs is the pharmacological reactivation of
4 latently infected T cells. We show here that a 4-deoxyphorbol ester derivative isolated
5 from *Euphorbia amygdaloides* ssp. *semiperfoliata*, 4*β*-dPE A, reactivates HIV-1 from
6 latency and could potentially contribute to decrease the viral reservoir. 4*β*-dPE A shows
7 two effects in the HIV replication cycle, infection inhibition and HIV transactivation,
8 similarly to other phorboids PKC agonists such PMA and prostratin and to diterpene
9 esters such SJ23B. Our data suggest 4*β*-dPE A is non-tumorigenic, unlike the related
10 compound PMA. As the compounds are highly similar, the lack of tumorigenicity by 4*β*-
11 dPE A could be due to the lack of a long side lipophilic chain that is present in PMA. 4*β*-
12 dPE activates HIV transcription at nanomolar concentrations, lower than the
13 concentration needed by other latency reversing agents (LRAs) such as prostratin and
14 similar to bryostatin. PKCθ/MEK activation is required for the transcriptional activity, and
15 thus, anti-latency activity of 4*β*-dPE A. However, CD4, CXCR4 and CCR5 receptors
16 down-regulation effect seems to be independent of PCK/MEK, suggesting the existence
17 of at least two different targets for 4*β*-dPE A. Further, NF-κb transcription factor is
18 involved in 4*β*-dPE HIV reactivation, as previously shown for other PKCs agonists. We
19 also studied the effects of 4*β*-dPE A in combination with other LRAs. When 4*β*-dPE A
20 was combined with another PKC agonists such as prostratin an antagonic effect was
21 achieved, while, when combined with an HDAC inhibitor such as vorinostat, a strong
22 synergistic effect was obtained. Interestingly, the latency reversing effect of the
23 combination was synergistic diminishing the EC_{50} value but also increasing the efficacy

showed by the drugs alone. Further, combination of 4*β*-dPE A with antiretroviral drugs as CCR5 antagonist, NRTIs, NNRTIs and PIs, showed a consistent synergistic effect, suggesting that the combination would not induce undesirable effects on antiretroviral therapy (ART). Finally, 4*β*-dPE A induced latent HIV reactivation in CD4+ T cells of infected patients under ART at similar levels than the tumorigenic phorbol derivative PMA, showing a clear reactivation effect. In summary, we describe here the mechanism of action of a new potent deoxyphorbol derivative as a latency reversing agent candidate to decrease the size of HIV reservoirs.

Keywords: HIV-1, latency reversing agents, proteinkinase C, viral reactivation, phorbol esters

1. INTRODUCTION

Antiretroviral therapy (ART) has significantly decreased mortality attributable to AIDS, reducing the viral load to undetectable levels in infected individuals and delaying disease progression. However, the persistence of latent HIV reservoirs, particularly in resting memory T CD4+ cells, is a major obstacle to HIV eradication. Although resting T cells do not allow viral replication, they are a source of HIV when activated [1]. This is also true for other pathogens, such as Ebola virus, found to persist in the so-called sites of immune-privilege [2, 3], or hepatitis B virus, human papillomavirus and most herpesvirus, that persist as latent infections using different mechanisms [4].

ART acts through interference with different stages of HIV replication cycle but most of antiretrovirals tackle viral proteins so they are unable to eliminate viral reservoirs. Thus, the search for new classes of antiretroviral drugs continues to be indispensable, because of ART long-term toxicity [5] and drug resistances [6]. Treatment with molecules able to induce HIV-1 transcription in resting T cells is a strategy of reservoir elimination named "shock and kill" or “kick and kill”. This strategy aims to tackle the latent reservoir stimulating viral replication in latently infected CD4+ T cells, while blocking the spread of infection with antiviral therapy [7].

The latency-reversing agents (LRAs) or anti-latency drugs will be the “shock” of this strategy. There are several kinds of LRAs including epigenetic modifiers, TLR (toll like receptors) agonists, TCR (T-cell receptors) activators, PI3K/Akt pathway modulators, NF-κB agonists, PKC (protein kinase C) agonists and HDAC (histone deacetylase) inhibitors [8]. PKC agonists (prostratin or bryostatin) and HDAC inhibitors (vorinostat,

1 panobinostat or romidepsin) are the most studied LRAs. Moreover, some clinical studies
2 have been performed with these drugs. Vorinostat [9], bryostatin [10], romidepsin [11]
3 and TLR7 agonists [12] have been tested in clinical trials, but the results obtained were
4 not convincing. Moreover, a cure may require an effective “kill” strategy beyond the
5 immunological response, and some efforts have been made involving immune system
6 modulation [13]. These studies include a vaccine based “kill”, as the romidepsin (shock)
7 plus a therapeutic vaccine Vacc-4x and rhGM-CSF (kill) study, showing a slight but non-
8 significant reduction of the reservoir size [14], or the RIVER study, including an LRA as
9 the “shock” (vorinostat) and two immunomodulatory vaccines as the “kill”, but results
10 were also disappointing [15]. There could be several causes for this failure: the method
11 of reservoir quantification underestimates or overestimates its size [16], the doses used
12 in the assay were too low to avoid potential toxicity or the low efficacy of the drugs
13 selected. Other approaches have been explored. CAR T cells engineered to express
14 broadly neutralizing anti-HIV antibodies have been found to reduce the size of the viral
15 reservoirs of the blood of infected individuals on antiretroviral therapy, although these
16 results are too preliminary [17].

17 PKC agonists can activate some isoforms of protein kinases C (PKC) by binding to its
18 regulatory C1 domain, mimicking the action of the physiological ligand diacylglycerol
19 (DAG) and inducing viral reactivation. Prostratin was the first PKC agonist studied as a
20 potential anti-latency agent [18]. Bryostatin, an antineoplastic drug agonist of PKC,
21 showed lack of efficacy in the reduction of the latent reservoir in clinical trials, probably
22 due to the low doses used in the assay to avoid toxicity since systemic concentrations
23 were found undetectable [10]. Clinically, bryostatin toxicity occurs at sub-efficacious

1 doses limiting its use as LRA. If this effect were on target, it would significantly limit the
2 entire class of compounds. In this sense, some diterpenes and phorboids obtained from
3 *Euphorbiaceae* have also been described as LRAs with antiviral properties and
4 reactivation activity. However, slight differences in their structure lead to lack of activity
5 or a PKC isoform preference, modulating their reservoir reactivating activity [19].
6 Moreover, the chemical structure of bryostatin is completely unrelated to phorboids and
7 thus, its toxic effects could be due to a completely different target *in vivo* although there
8 isn't data about that. Identification and characterization of new phorboids with different
9 PKC binding profile, identification of other potential targets of these drugs and selection
10 of non-tumorigenic phorboids with low toxicity could be helpful to address their efficacy
11 as LRAs and toxicity.

12 Finally, as in ART, reactivation of the latent reservoir could only be achieved by the
13 combination of different drugs or antibodies able to diminish doses, and thus toxicity,
14 and to achieve cell activation enough to eliminate the reservoirs [20, 21]. In this sense, it
15 is important to point out that this therapy is intended to be used in combination with
16 ART. Some previous reports have shown that antiretroviral drugs could modulate the
17 LRAs activity and, conversely, LRAs can affect the antiviral activity of ART. For example,
18 some protease inhibitors diminish the viral reactivation obtained with LRAs [22].

19 We have reported previously the antiviral activity of 4-deoxyphorbol derivative
20 compounds isolated from *Euphorbia amygdaloides* ssp. *semiperfoliata* [23]. In the
21 present study, the mechanism of action and cellular pathways involved in the latency-
22 reversal and anti-HIV-1 effects of 4*β*-dPE A*,* have been evaluated. This compound
23 shows antiviral activity through down-regulation of receptors and is able to induce HIV

reactivation. This class of compounds could be used as an adjuvant therapy to eliminate the latent reservoirs of HIV-1.

2. MATERIAL AND METHODS

2.1. Reagents

4*β*-dPE A was isolated from *Euphorbia amygdaloides* ssp. *semiperfoliata* and provided by CNRS (Centre National de la Recherche Scientifique CNRS, France). The compound was solubilized in DMSO to a final concentration of 10 mM and stored aliquoted at -80°C. Monoclonal antibodies (mAbs) to CD4, CXCR4 and CCR5 were supplied by Becton Dickinson (Mountain View, CA, USA). Interleukin-2 (IL-2) was supplied by Chiron (Emeryville, CA. USA). The inhibitor rottlerin was obtained from Alexis Co. (Lausanne, Switzerland) and the inhibitors Gö6850 and Gö6976 were obtained from Calbiochem (EMD Biosciences, Inc. Darmstadt, Germany). Renilla and Luciferase Assay Systems and CellTiter Glo viability assay were supplied by Promega (Madison, WI, USA). DMSO and MEK1/2 inhibitor PD184352 were supplied by Sigma-Aldrich (St Quentin-Fallavier, France). The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: SAHA (vorinostat), raltegravir, lamivudine, tenofovir, emtricitabine, abacavir, efavirenz and ritonavir. Prostratin, PMA and bryostatin-1 were purchased from Sigma-Aldrich (St Louis, MO).

2.2. Cells

MT-2 cells (American Type Culture Collection, Manassas, VA, USA, Ref: CRL-2560) and Jurkat 5.1 LTR-Luc cells (obtained from A. Israel, Institute Pasteur, Paris, France)

1 were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 2 mM L-
2 glutamine, penicillin (50 IU/ml) and streptomycin (50 mg/ml) (all Whittaker M.A. Bio-
3 Products, Walkerville, MD, USA) and split twice a week. The Jurkat 5.1 LTR-Luc cell line
4 is a Jurkat-derived clone stably transfected with a plasmid containing the luciferase gene
5 driven by the HIV-1-LTR promoter and was maintained in complete medium
6 supplemented with G418 (200 ug/ml). 293T cells, obtained from the American type
7 culture collection ATCC ref: ATCC® CRL-3216™, and TZM-bl cells (cell line stably
8 transfected with an LTR-Luc), obtained through the NIH AIDS Reagent Program,
9 Division of AIDS, NIAID, NIH: TZM-bl cells (Cat#8129) from Dr. John C. Kappes, and Dr.
10 Xiaoyun Wu, were both cultured in DMEM medium containing 10% (v/v) fetal bovine
11 serum, 2 mM L-glutamine, penicillin (50 IU/ml) and streptomycin (50 mg/ml) (Whittaker)
12 and split twice a week. PBMCs (peripheral blood mononuclear cells) were isolated from
13 healthy blood donors by centrifugation through a Ficoll-Hypaque gradient (Pharmacia
14 Corporation, North Peapack, NJ) and were suspended in RPMI 1640 medium
15 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (100
16 mg/ml streptomycin and 100 U/ml penicillin) (all Whittaker M.A. Bio-Products,
17 Walkerville, MD, USA) before culture at a concentration of $2x10^6$ cells/mL. In some
18 experiments, PBMCs were activated with IL-2 (300 IU/mL) for at least 48 h. Cells were
19 cultured at 37 °C in a 5% CO_2 humidified atmosphere. Proper informed consent was
20 obtained from each subject in accordance with the Spanish legislation on blood donor
21 regulations. Confidentiality and privacy were assured.

22 **2.3. PBMCs from HIV infected patients**

23 PBMCs of ART infected patients were obtained from Dra. Sonsoles Sanchez Palomino,
24 from the Clinic Hospital, Barcelona, Spain. Written informed consent was obtained from

all participants. CD4 T cells were purified by positive selection using human CD4+ T Cell Isolation Kits and MS columns (both Miltenyi biotech, Bergisch Gladbach, Germany). CD4+ T cells were then cultured at 37 °C in a 5% CO_2 humidified atmosphere in complete medium until use.

2.4. Plasmids

The vector pNL4.3-luc was generated by cloning the luciferase gene in the HIV-1 proviral clone pNL4.3 [24]. Plasmid pNL4.3-Ren was generated cloning the renilla gene in the *nef* site of pNL4.3 and pJR-Ren plasmid was generated cloning the *env* gene of HIV-1 JR_{CSF} in the pNL4.3-Ren plasmid [25]. pNL4.3-Δenv-Luc was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3.Luc.R^{-}.E^{-} from Dr. Nathaniel Landau and pcDNA-VSV encoding the G protein of VSV from Dr. Arenzana-Seisdedos from Pasteur Institute [26]. The 3-enh-κB-ConA-luc plasmid carries a luciferase gene under the control of three synthetic copies of the κB consensus of the immunoglobulin k-chain promoter cloned into the BamHI site located upstream from the conalbumin transcription start site [27]. The LTR-Luc plasmid carries a luciferase gene under the control of HIV-1- LTR region [28]. The expression construct pNFAT-LUC containing three tandem copies of the distal NFAT-binding site of the IL-2 gene promoter coupled to the IL-2 minimal promoter was kindly provided by Dr. Juan Miguel Redondo (National Center for Cardiovascular Research, Madrid, Spain) [29].

Plasmids were amplified transforming *E. coli* DH5α cells and cultured in LB supplemented with ampicillin at 37°C for 18 hours. All plasmids were purified using Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) following the manufacturer

instructions. DNA concentration was determined in a Nanodrop (Thermoscientific, Waltham, MA, USA).

2.5. Viruses

JR-Ren (HIV-1 R5 tropic) and NL4.3-Ren (HIV-1 X4 tropic) viral supernatants were obtained from transfection of pJR-Ren and pNL4.3-Ren plasmids. HIV-VSV (NL4.3-∆env-VSV-Luc), HIV-1 with a luciferase reporter gene in the position of *nef* and expressing VSV G protein instead of HIV-1 env gene, was obtained by co-transfection of pNL4.3-∆env-Luc and pcDNA-VSV encoding the G protein of VSV. Calcium phosphate transfection of plasmids in 293T cells was used to obtain viral supernatants.

2.6. Anti-HIV-1 activity evaluation by recombinant virus assay

The initial evaluation of 4*β*-dPE A was performed in MT-2 cells infected with NL4.3-Ren obtained previously by transfection in 293T cells. The assay was performed in 96 well microplate seeded with 100,000 cells/well in a final volume of 200 µl. Cell cultures were infected with viral supernatants (100,000 RLUs/w) and after 48 hours cell pellets were lysed and luciferase activity measured in a luminometer (Berthold detection systems, Pforzheim, Germany). X4 (NL4.3-Ren) or R5 (JR-Ren) infections of IL-2 activated PBMCs followed the same procedure. In the same way, HIV-VSV (NL4.3-∆env-VSV-Luc) and HIV-1 (NL4.3-Ren) were used to infect MT-2 cells in the presence of different concentrations of 4*β*-dPE A to assess the effect of compounds in HIV-1 entry. All the experiments were controlled with cells treated with the same DMSO concentration of 4*β*-dPE A. HIV-1 replication inhibition was evaluated by measuring the luminescence activity or RLUs, with 100% being the infection of non-treated cells. IC_{50} was calculated with a non-linear regression formula using GraphPad Prism software.

1 **2.7. Toxicity evaluation**

2 Cell toxicity was evaluated by treating mock infected cells with the same concentrations
3 of 4*β*-dPE A or LRAs (PMA, bryostatin-1, prostratin and vorinostat) used in the antiviral
4 assays or with higher concentrations of 4*β*-dPE A and other LRAs (PMA, bryostatin-1,
5 prostratin and vorinostat) or its combination with LRAs or antiretroviral drugs. After 48
6 hours in culture, CellTiter Glo reagent (Promega, Madison, WI, USA) was added and
7 cell viability measured by RLUs quantification in a luminometer. Cell viability is
8 expressed as percentage of viable cells compared to a non-treated (DMSO same
9 concentration as compound) control (100%). Long-term toxicity evaluation at different
10 times was performed with 4*β*-dPE A in IL-2-activated-PBMCs for 14 days. CC_{50} was
11 calculated with a non-linear regression formula using GraphPad Prism software.

12 **2.8. Evaluation of viral transcription**

13 MT-2 cells or resting PBMCs (in culture for at least 24 hours) were suspended in 350 μL
14 of RPMI without supplements and electroporated using an Easyject plus Electroporator
15 (Equibio, Middlesex, UK) at 260 (MT-2) or 320 (PBMCs) V, 1500 mF and maximum
16 resistance with 1μg/10^6 cells of a luciferase plasmid under the control of the whole
17 genome of HIV-1 (NL4.3-Luc). Afterwards MT-2, resting PBMCs and Jurkat 5.1-LTR-Luc
18 or TZM-bl (cell lines stably transfected with an HIV-1 LTR-Luc) cells were seeded in 24
19 well microplates and treated with different concentrations of 4*β*-dPE A or PMA (0.1 μM),
20 as a reference control of HIV-1 transactivation, and left in culture in complete RPMI at
21 37°C. 48 hours later, cultures were lysed with luciferase buffer and RLUs measured in a
22 luminometer. This procedure was also used for the transfection of luciferase plasmids
23 under the control of transcription factors NFAT (NFAT-Luc) or NF-κB (NF-κB-luc) or the

long terminal repeat of HIV (LTR-Luc) in resting PBMCs to evaluate transcriptional activity. The transcriptional activity of 4*β*-dPE A in resting PBMCs was also studied in the presence of PKCs inhibitors Gö6850 (Bisindolylmaleimide 1 μM), Gö6976 (1 μM) or rottlerin (3 μM) and MEK inhibitor PD184352 (1 μM).

2.9. Receptor expression kinetics

Single-, double- or three-color immunophenotyping was performed with a FACScalibur flow cytometer using CD4 (clone SK3), CXCR4 (clone 12G5) and CCR5 (clone 2D7/CCR5) antibodies conjugated to the appropriate fluorochrome, all obtained from Becton Dickinson (Heidelberg, Germany). Background staining was assessed with the appropriate isotype- and fluorochrome-matched control mAb and subtracted. Activated PBMCs or MT-2 were treated with different concentrations of 4*β*-dPE A for 48, 24 and 2 hours. Cells were then collected and CD4, CXCR4 and/or CCR5 receptors expression were evaluated. The effect of PKCs inhibitors Gö6850 (bisindolylmaleimide), rottlerin and MEK inhibitor PD184352 was also studied in the expression of CD4, CXCR4 and CCR5 receptors in IL-2 activated PBMCs. The analysis was performed using forward-versus-side scatter dot plots. Results are shown as percentage of receptor fluorescence mean intensity (%MIF) obtained by FlowJo 8.0 software.

2.10. Immunofluorescence Assays

For immunofluorescence assays, resting PBMCs were incubated in a μ-Slide 8 well ibiTreat (Ibidi GmbH) for 48h, in the presence or absence of PMA, 4*β*-dPE A (100 nM) or DMSO (vehicle). Then, they were permeabilized with the cell fixative CytoskelFix (Cytoskeleton) for 4 min at -20ºC. After washing twice with 0.1% glycine/PBS, cells were

incubated with phospho-PKCθ (Thr538) (Cell Signaling Technology, Danvers, MA) and Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes) antibodies. Washes were performed with 1X PBS. 4′,6-diamidino-2-phenylindole (Dapi) was used for nuclear staining. Images were obtained with Leica DMI 4000B Inverted Microscope (Leica Microsystems, Wetzlar, Germany). Mean fluorescence intensity (MI) was calculated using Image J software and values are represented in bar diagrams showing statistical significance.

2.11. Combination experiments

4*β*-dPE A was tested as a latency reversing agent alone and in combination with other latency reversing agents as prostratin and vorinostat and a single concentration of antiretrovirals abacavir (ABC 1 μM), efavirenz (EFV 0.01 μM), ritonavir (Rito 0.1 μM), lamivudine (3TC 1 μM), emtricitabine (FTC 1 μM) and tenofovir (TFV 1 μM). The model used to evaluate the anti-latency effect was performed in primary PBMCs isolated from healthy donors. Briefly, resting PBMCs were transfected with a recombinant HIV-1 (NL4.3-Luc) and treated with serial dilutions or single concentrations or the drugs to be tested. After 48 hours, cell culture was lysed and RLUS were obtained in a luminometer. A control of the vehicle used for the dilution of the drugs was used for all the concentrations tested (100%). For LRAs, EC_{50} and maximum effect (Emax) were calculated for each drug separately and EC_{50}/EC_{50} ratios were determined. Afterwards, combinations experiments were performed following the same procedure with the drugs alone as compared to their EC_{50}/EC_{50} combination. For antiretrovirals-LRA combinations, a t-test analysis was performed between the reactivation rates obtained with PMA or 4*β*-dPE A alone and the combination with the antiretroviral drug.

Anti-HIV-1 activity of 4*β*-dPE A was also evaluated in combination with antiretrovirals such as the nucleoside analogue reverse transcriptase inhibitors (NRTI) lamivudine, emtricitabine and abacavir, the nucleotide analogue reverse transcriptase inhibitor (NtRTI) tenofovir, the non-nucleoside analogue reverse transcriptase inhibitor (NNRTI) efavirenz and the protease inhibitor (PI) ritonavir. Briefly, MT-2 cells were pre-treated with serial dilutions of the drugs to be tested for 30 minutes and infected with a recombinant HIV-1 (NL4.3-Ren). After 48 hours, cell culture was lysed and RLUS were obtained in a luminometer. A control of the vehicle used for the dilution of the drugs was used for all the concentrations tested (100%). IC_{50} was calculated for each drug separately and IC_{50}/IC_{50} ratios were determined. Afterwards, combinations experiments were performed following the same procedure with the drugs alone as compared to their IC_{50}/IC_{50} combination.

2.12. Transformation assays

NIH 3T3 fibroblasts were maintained in medium DMEM supplemented with 10% calf serum and treated either with SJ23B, prostratin, or PMA. All compounds were used at 0.1 mM concentration. As positive control, K-Ras V12 (pCEFL-KZ-HAK-Ras V12) was transfected by the calcium phosphate precipitation technique. As negative control, vector pCEFL-KZ-HA was transfected under similar conditions. Morphologically transformed foci were scored after 2–3 weeks in culture [30]. Cells were washed with PBS 1x, fixed with PFA 4% for 30 min, washed again with deionized water and stained with Giemsa staining solution. Transformed cell foci appeared intense blue colored.

2.13. Anti-latency effect of 4*β*-dPE A in CD4 T cells from infected patients on ART

CD4 T cells from 3 infected patients were cultured in the presence of PMA 100 nM or 4*β*-dPE A 100 nM or left untreated for 48 hours. Cells were then collected and

supernatants were submitted to viral load quantification using the kit Aptima HIV-1 Quant Dx (Hologic, Gent, Belgium). Data obtained were analyzed using Graph Pad Prism software, two-way ANOVA.

2.14. Statistics

FlowJo 8.0 software was used to analyze flow cytometry data and results are shown as percentage of receptor fluorescence mean intensity (%MIF) as compared to a non-treated control (100%). GraphPad Prism software was used to calculate effective concentration 50%, inhibitory concentration 50%, cytotoxic concentration 50% (EC_{50}, IC_{50}, CC_{50}) and Emax (top) in all the experiments (Non-linear regression, dose-response stimulation, log[agonist] vs response for anti-latency experiments and dose response inhibition, log[inh] vs response for antiviral experiments) and to represent concentration-response curves. Confidence intervals 95% (CI95%) and R^2 are provided for all the calculated concentrations. GraphPad Prism was also used to perform ANOVA or t-test analysis to determine the value of p (* $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$). Finally, the Calcusyn Software was used to evaluate combination index (CI) for each combination. All the experiments were performed at least in triplicates in three independent experiments.

3. RESULTS

3.1. 4*β*-dPE A inhibits *in vitro* HIV infection with low cytotoxicity and activates viral transcription

4*β*-dPE A is a phorbol derivative with special chemical characteristics, as the lack of the oxygen at position 4 (figure 1) and thus, we aimed to study its potential antiviral and

latency reversing activity. To that end, first we used MT-2 cells infected with an X4 tropic recombinant virus (NL4.3-Ren) in the presence of different concentrations of 4*β*-dPE A. A strong inhibition of viral replication was observed at nanomolar concentrations, with an IC_{50} value of 0.64 nM. Further, when IL-2 activated PBMCs were infected under the same conditions with X4 or R5 tropic HIV, a stronger inhibition of viral replication was obtained, with IC_{50} values of 0.31 and 0.24 nM, respectively (Figure 2A and table 1). On the other hand, when a VSV pseudotyped HIV-1 was used to evaluate if the effect of the compound was HIV receptors dependent, an incomplete curve of inhibition was obtained, with an IC_{50} value of around 100 nM (table 1), suggesting that, as other phorbol derivatives, viral entry is one of the main targets of HIV inhibition, although other targets could be involved.

Regarding toxicity, cell death was not observed at the concentrations tested (10000 nM). However, since toxicity is a cause of concern for anti-latency drugs, we further evaluated 4*β*-dPE A toxicity in a long-term viability assay (14 days) in IL-2 activated PBMCs at concentrations of 0.1, 1 and 10 μM. Cell viability was determined at different times along treatment. As observed in figure 2A, 4*β*-dPE A was not toxic to human PBMCs even at 14 days of treatment.

We also compare the antiviral activity of 4*β*-dPE A with other LRAs as prostratin, vorinostat, bryostatin-1 and the tumorigenic phorbol ester PMA (figure 2B and table 2). We found that all of them inhibited HIV-1 infection in both, MT-2 cells and IL-2 activated PBMCs. Prostratin and vorinostat were the less powerful inhibitors with IC_{50} of 100-200 and 40-70 nM, respectively, while bryostatin 1 and PMA showed lower IC_{50} values (MT-2 cells 0.032 and 0.069 for bryostatin-1 and PMA, respectively; and PBMCs 0.17 and

1 0.18 nM for bryostatin-1 and PMA, respectively) in the range of 4*β*-dPE A (figure 2B and
2 tables 1 and 2). None of the LRAs showed cell toxicity at the concentrations tested.
3
4 **3.2. 4*β*-dPE A induces HIV transcription**
5 The very low number of latently HIV-infected cells *in vivo* makes purification and
6 biochemical analysis of these cells hard to achieve. Moreover, several studies show that
7 *in vitro* assays used to quantify the reservoir from PBMCs display a large range
8 depending on the technique used [31]. Therefore, we have used several methods to
9 evaluate the transcriptional effect of these drugs. First, we have used a model based on
10 MT-2 cells and primary human resting PBMCs transfected with luciferase plasmids
11 under the control of HIV-1 whole genome (NL4.3-Luc). We have also tested
12 transcriptional activation in non-infected TZMbl and Jurkat 5.1 LTR-Luc cells. These
13 cells lines do not need HIV transfections since they contain in its genome integrated
14 copies of the luciferase gene under the control of the HIV-1 promoter LTR. 4*β*-dPE A
15 activates HIV transcription in all the scenarios tested, inducing high levels of HIV-1
16 transcription with nanomolar concentrations (figure 3A).
17 Second, we have calculated the EC_{50} values in HIV transcription in PBMCs. As it can be
18 seen in figure 3B, all the compounds tested showed viral reactivation in resting PBMCs.
19 As shown in table 3, 4*β*-dPE A displayed an EC_{50} value of around 2.9 nM as
20 transcriptional activator, which is at least 70 and 480 fold lower than the EC_{50} of
21 vorinostat and prostratin, respectively, although it was less potent than PMA (EC_{50} 0.031
22 nM). In the same assay, bryostatin displayed an EC_{50} value of 3.4 nM, which is almost
23 the same of 4*β*-dPE A. However, due to compound availability, we only reach

concentrations of 100 nM with bryostatin and cell toxicity was not reached. Therefore, CC_{50} of bryostatin was higher than 100 nM with an SI higher than 21, while 4*β*-dPE A was higher than 10000 (figure 3 and table 3).

To assure that the effect of the compounds was not impaired by their potential toxicity, all the compounds were subjected to cell toxicity evaluation in parallel with the same concentrations used in the assay. We found no toxicity at the concentrations tested for all the compounds in PBMCS, MT-2, TZMbl or Jurkat 5.1 cell lines (data from figure 2 or not shown).

3.3. 4*β*-dPE A decreases the expression of HIV-1 entry receptors

Viral entry involves the interaction of HIV gp120 and gp41 with CD4, CXCR4 and/or CCR5 receptors. It has been described previously the down regulation of these receptors by PKC agonists such as prostratin [32] and SJ23B [19]. Therefore, expression of cell surface receptors by three color immunostaining was measured by flow cytometry in MT-2 cells and IL-2 activated PBMCs. Cell cultures were stimulated with PMA or 4*β*-dPE A and left in culture for 2, 24 and 48 h (Figure 4).

4*β*-dPE A down-regulates CD4, CXCR4 and/or CCR5 expression in MT-2 cells and IL-2 activated PBMCs, in a concentration-dependent manner. 4*β*-dPE A 1nM was not effective, but 10 and 100 nM concentrations were enough to down-regulate receptors with a pattern of down-regulation similar to that as PMA at 100 nM (Figure 4).

3.4. PKCθ/MEK pathway is required for 4*β*-dPE A HIV transcriptional activity

To assess the PKC/MEK dependence of 4β-dPE A-mediated antagonism of HIV latency, resting PBMCs were treated with PKCs chemical inhibitors Gö6976 1 μM (classical PKCs inhibitor), bisindolylmaleimide 1 μM (Gö6850, classical and novel PKCs inhibitor), rottlerin 3 μM (PKCθ inhibitor) or MEK inhibitor PD184352 (IMEK) 1 μM and HIV transcriptional activity of 4β-dPE A was also evaluated (Figure 5). We have used the more target specific concentrations for all the drugs based on previous papers [33, 34].
The use of a classical PKC inhibitor did not show an inhibitory effect in the transcriptional activity of 4β-dPE A. On the other hand, the inhibition of classical and novel PKCs resulted in impairment of this effect, suggesting that it could be mediated through novel PKCs. Therefore, we have selectively inhibited PKCθ (rottlerin 3 μM) and we found that the transcriptional activity was also blocked, and thus, PKCθ seems to be strongly involved in the transcriptional response to the deoxyphorbol. Further, since PKC activation could lead to the activation of MEK-RAF pathway of cellular growing, we also studied the effect of MEK inhibition in the transcriptional activity of 4β-dPE A. As shown in figure 4, MEK inhibition decreased transcriptional activity induced by 4β-dPE A and PMA, suggesting an effect PKCθ/MEK dependent.

3.5. PKCθ/MEK pathway is not involved in 4β-dPE A activity on receptors down-regulation

To study the specific PKC isoform involved in 4β-dPE A receptor down-regulation, we treated IL-2 activated PBMCs with PKCs chemical inhibitors, bisindolylmaleimide or rottlerin, and we evaluated the 4β-dPE A effect on CD4, CXCR4 and CCR5 receptor expression. Downstream PKC signaling in T cells may involve Raf-Mek dependent

activation of MAPKs, and thus MEK inhibition could also play a role in the 4β-dPE A effect. Therefore, PBMCs were treated with MEK inhibitor PD184352 and the effect of 4β-dPE A in the surface cell receptor expression was also measured.

We found that the inhibition of PKC or MEK did not modify the expression of CD4, CXCR4 or CCR5 receptors with or without 4β-dPE A treatment, suggesting that PKC/MEK pathway is not related to the down-regulation of receptors exerted by 4β-dPE A, and thus, to the anti-HIV activity. Further, the differences of receptors expression between PKC inhibitor treated cells, MEK inhibitor treated cells and cell treated with no inhibitor were not significant, suggesting that these inhibitors did not affect the receptor expression by themselves (Figure 6).

3.6. 4β-dPE A induces a different pattern of intracellular distribution and phosphorylation of PKCθ

Since novel PKCs, and specifically PKCθ, seems to be involved in the transcriptional response to 4β-dPE A, we have analyzed its effect in PKCθ activation. Moreover, PKCθ is the putative target of PKC agonists in T cells and it is critical in the regulation of T-cell activation, proliferation and differentiation showing high levels in T cells [33]. Therefore, we have analyzed the PKCθ phosphorylation in T538 after the treatment with LRAs by immunofluorescence microscopy. Figure 7 shows the results of PKCθ phosphorylation and cellular localization in response to PMA or 4β-dPE A treatment compared to an untreated control.

Results show a different pattern of intracellular distribution of PKCθ when PMA (used as pan PKC agonist control) or 4β-dPE A are used. These results do not show a clear

PKCθ membrane translocation but an increase in phosphorylation, as quantitation analysis shows, and an altered cellular localization of PKCθ, as previously reported by other groups [35], suggesting this PKC isoform is a cellular target in PBCMs of 4*β*-dPE A.

3.7. NF-κB transcription factor is involved in 4*β*-dPE A transcriptional activity

To further analyze the mechanism of 4*β*-dPE A reactivation activity, we studied the effect on transcriptional factors crucial for HIV-1 transcription (NF-κB) and for T-cell activation (NFAT), since the induction of HIV transcription without a massive T cell activation would be desirable.

To that end, luciferase expression vectors under the control of three tandem κB consensus repeats (NF-κB Luc), NFAT (NFAT Luc) or LTR region of HIV (LTR Luc) were transfected in resting PBMCs, treated with 4*β*-dPE A at three different concentrations (1, 10 and 100 nM) or with PMA (100 nM) and luciferase activity was measured after 48 h in culture (figure 8).

As observed in figure 8, 4*β*-dPE A induced the activation of the HIV LTR and NF-kB transcription factor with the highest concentration of 100 nM as PMA. However, NFAT was not influenced by 4*β*-dPE A or PMA treatment, while Ionomycin (Io) strongly increased its expression. These data suggest that the transcriptional activity of PKCs agonist is quite selective depending on NF-κB but not on NFAT.

3.8. 4*β*-dPE A displays a synergistic effect when combined with vorinostat but not with prostratin

Since 4*β*-dPE A is a PKC agonist, we tested its combination with another PKC agonist, prostratin, and with a histone deacetylase inhibitor (HDACi), vorinostat. Combinations were evaluated among all the three drugs, including 4*β*-dPE A/prostratin, 4*β*-dPE A/vorinostat and prostratin/vorinostat and the combination effect on EC_{50} and Emax was studied. Combination index (CI) has been calculated for all the combinations following the formula of Chou & Talalay. In this paper, CIs greater than 1,30 indicate antagonism, CIs between 1.10 and1.30 weak antagonism, CIs between 0.90 and 1.10 and additive effect, CIs between 0.70 and 0.90 weak synergy and CIs of less than 0.70 strong synergy.

The combination of 4*β*-dPE A with prostratin showed a highly antagonistic effect since its CI was of 16.65 although it showed only a clear antagonistic tendency toward lowering of Emax value. On the other hand, combination of 4*β*-dPE A with vorinostat showed a strong synergism, with a CI value of 0.20 as well as the combination of prostratin with vorinostat with a CI of 0.21 (Figure 9). These results show that the combination of 4*β*-dPE A with other PKC agonist as prostratin increase the need of concentrations needed to obtain the same transcriptional activating effect, while when combined with an HDAC inhibitor as vorinostat, the situation is completely different, decreasing the need of concentration and obtaining a synergic effect. Moreover, when vorinostat and 4*β*-dPE A are combined, the efficacy of transcriptional activation increases to almost double the effect of the drugs alone.

3.9. 4*β*-dPE A displays a strong synergistic antiviral effect in combination with NRTIs, NtRTIs, NNRTIs and PIs

1 Since the LRA treatment is designed to be administered together with ART, we
2 evaluated the effect of 4*β*-dPE A in the antiviral effect of antiretroviral therapy.
3 Therefore, combination experiments with different types of antiretroviral drugs were
4 performed. To that end, the entry inhibitor maraviroc (MVC), the nucleoside analogue
5 reverse transcriptase inhibitors (NRTI) lamivudine, emtricitabine and abacavir, the
6 nucleotide analogue reverse transcriptase inhibitors (NtRTI) tenofovir, the non-
7 nucleoside analogue reverse transcriptase inhibitor (NNRTI) efavirenz and the protease
8 inhibitor (PI) ritonavir were combined with 4*β*-dPE A using IC_{50}/IC_{50} ratios (Figure 10).
9 We have found that the anti-HIV effect of all the antiretroviral drugs tested in the
10 presence of 4*β*-dPE A showed a strong synergism with CIs below 0.70 for all the
11 concentrations tested, diminishing the concentrations needed to inhibit HIV infection.
12 However, the combination 4*β*-dPE A/efavirenz showed only a weak synergism with a CI
13 of 0.78 and the combination with 4*β*-dPE A/maraviroc was less synergic when
14 concentrations were higher (ED90) (Figure 10).
15

16 **3.10. Antiretroviral drugs did not modify the reactivation capacity of 4*β*-dPE A**

17 To test whether antiretroviral drugs could have an effect on the reactivation capacity of
18 4*β*-dPE A, a single concentration combination experiment was performed (figure 11).
19 Concentrations were selected as follows: we have used the EC_{90} obtained from
20 reactivation experiments of 4*β*-dPE A and PMA and the IC_{90} obtained from infection
21 experiments of antiretroviral drugs, since we found no reactivation effect of antiretroviral
22 drugs alone.
23 There were no significant differences among the rate of reactivation of PMA or 4*β*-dPE A
24 alone and the rate of reactivation of the combination with an antiretroviral drug. Only

abacavir showed a slight tendency to increase the viral reactivation exerted by PMA and 4*β*-dPE A, but it was still not significant.

3.11. 4*β*-dPE A does not induce cell transformation

A major cause of concern of PKC agonists is their potential tumor-promoting activity. However, 4*β*-dPE A lacks a key hydroxyl that was thought to be required for PKC activation although nothing is known about its involvement in tumor-promoting activity. Besides, 4-deoxyphorbols lack the long and lipophilic side chain of tumor-promoter PKC agonist PMA, which would suggest a different intracellular activity. To rule out the potential tumor-promoting activity of 4*β*-dPE A we performed transforming cell assays in NIH 3T3 fibroblasts.

We found that both 4*β*-dPE A and prostratin were unable to elicit transforming foci, while transfection with KrasV12 strongly induced cell transformation. Further, PMA treatment induced foci formation but to a lesser extent than KrasV12 (Fig. 12).

3.12. 4*β*-dPE A reactivates HIV-1 fin CD4+ T cells from infected patients receiving ART

Cell lines and transfected PBMCs are models to evaluate the transcriptional activity of compounds. However, although Jurkat 5.1 LTR-Luc and TZMbl cells contains an integrated copy of the LTR of HIV-1, the assay is not performed in real latently infected cells. To confirm that 4*β*-dPE A activates HIV-1 transcription in latently infected T cells, CD4+ lymphocytes obtained from 3 infected patients were treated with a fixed and effective concentration of 100 nM of 4*β*-dPE A and compared to the same concentration

of PMA. In this experiment, the effect of the compounds is assessed directly in latent infected cells, since ART does not allow viral replication.

Our results show that 4*β*-dPE A reactivates HIV from latent infected cells with a concentration of 100 nM, as well as PMA. This fact suggests that 4*β*-dPE A could be effective in reactivating the viral reservoir *in vivo*.

4. DISCUSSION

The identification of potent natural or synthetic PKC agonists lacking tumor-promoting activity and cellular proliferative activities has opened new research avenues for the treatment of cancer and HIV-1 latency. We show here that 4*β*-dPE A (12-*O*-Tigloyl-13-*O*-isobutyroyl-20-hydroxyl-4*β*-deoxyphorbol), a 4-deoxyphorbol ester derivative isolated from *Euphorbia amygdaloides* ssp *semiperfoliata*, reactivates HIV-1 from latency *ex vivo*.

4*β*-dPE A showed two effects in the HIV replication cycle. First, it inhibits HIV infection, reported previously by us [23] and secondly it induces HIV transcriptional activation. These effects have been described for other PKC agonists such as phorbol 12-myristate 13-acetate (PMA), bryostatin [36], prostratin [32] or SJ23B [19]. However, PMA, a phorbol diester, induced activation of HIV-1, but its potent tumor promoting activity turns its therapeutic use unacceptable [37]. Prostratin, bryostatin and SJ23B are PKC agonists, analogs of diacylglicerol (DAG), but they lack the tumorigenic activity of phorbol esters as PMA. Bryostatin is chemically unrelated to phorbol esters since it is a polyacetylated macrolactone developed as an anti-tumoral agent. It can be hypothesized that the antitumor activity of PKC agonists in general could be related to a

1 high PKC activation leading to its degradation by the proteasome, while the natural
2 ligand DAG or phorbol esters as PMA, with long side chains, will be inactivated before
3 PKC degradation, promoting cellular growth. Moreover, it has been shown that phorbol
4 esters with long acyl chain(s) such as the PMA, induced a distinct intracellular
5 translocation pattern of PKC and that this activity rapidly decreased with a shortening of
6 the acyl side chain [38].

7 Thus, just like prostratin, 4*β*-dPE A, lacking the long side chain, should not display this
8 tumor promoting activity. In fact, we did not find long term toxicity after a 2-week
9 treatment of PBMCs (figure 2A) and the transformation assay confirmed the lack of
10 tumor-promoting activity (figure 12). Short term toxicity experiments *in vitro* supported
11 the low toxicity of 4*β*-dPE A, since CC_{50} was not reached at least at 10 µM in PBMCs
12 and MT-2 cells, displaying a high specificity as HIV inhibitor with a specificity index
13 greater than 1500 and 30000 for MT-2 cells and PBMCs, respectively (Table 1 and
14 figure 2A).

15 Therefore, we have studied the effect of this compound in the HIV-1 replication cycle at
16 non-toxic concentrations. First, we evaluated the HIV-1 inhibition activity in MT-2 cells
17 and IL-2 activated PBMCs. IC_{50} values of 4*β*-dPE A were between 0,3 and 0,6 nM while
18 PMA and bryostatin showed an IC_{50} value in the same range in MT-2 but more powerful
19 in MT-2 cells (0.18 and 0.17 nM in PBMCs and 0.069 and 0.032 in MT-2, respectively).
20 We also found that 4*β*-dPE A was 200-600 times more powerful than prostratin as an
21 HIV infection inhibitor (IC_{50} 132.6 and 204.6 nM in MT-2 cells and PBMCs, respectively)
22 and 100-140 than vorinostat (IC_{50} 68.25 and 43.41 nM in MT-2 cells and PBMCs,
23 respectively).

1 This anti-HIV activity was in part due to the down-regulation of CD4, CXCR4 and CCR5,
2 as our data of VSV-HIV infection and down-regulation or receptors suggest (figure 2A
3 and 4). These data show that 4*β*-dPE A is at least as effective as SJ23B in receptor
4 down-regulation and more effective than prostratin.
5 Among PKC agonists, only bryostatin entered clinical trials as HIV anti-latency agent,
6 although results were disappointing. The reasons of this failure are not clear, but in vivo
7 toxicity of bryostatin could be behind it. In fact, clinical assays with bryostatin were
8 designed with the lowest doses possible to avoid the potential toxicity of PKC agonists.
9 However, clinical assays did not show any effect on the transcription of latent HIV, due
10 probably, as the authors stated, to low plasma concentrations [10]. However, as we
11 stated in the introduction, the chemical structure of bryostatin is completely different to
12 phorboids and thus, its toxicity *in vivo* could be result of the interaction with targets not
13 related to PKCs.
14 Therefore, we have studied the effect of 4*β*-dPE A as a transcriptional reactivator and
15 anti-latency drug. We show that it is a powerful HIV transcriptional activator agent, with
16 *in vitro* active concentrations in the low nM range in MT-2, TZMbl and Jurkat 5.1 LTR-
17 Luc cell lines and in resting PBMCs. Indeed, 100 nM concentration in all the cell lines
18 was enough to induce HIV transcription at higher levels than PMA at the same
19 concentration (figure 3A). In resting PBMCs though, it showed an EC_{50} of 2.9 nM, in the
20 same range than bryostatin (3.4 nM) but 100 fold higher than PMA (0.031 nM) (figure 3B
21 and table 3).
22 On the other hand, 4*β*-dPE A was more powerful as transcriptional activator than
23 vorinostat and prostratin with EC_{50} values around 250 and 1600 fold lower, respectively,

1 confirming the high potency showed previously in the infectious experiments and its high
2 specificity (specificity index is greater than 10000. Figure 3B and table 3).
3 The putative target of this class of compounds are PKCs. Classical PKCs (cPKCs) and
4 novel PKCs (nPKCs) have two tandem C1 domains in their N-terminal domain, the C1a
5 and C1b domains, which show high binding affinities for DAG, phorbol esters and other
6 PKC activators. Interestingly, phorbol esters binding sites in PKC C1 domain have been
7 described and chemical pharmacophores identified. These pharmacophores included a
8 triad of oxygenation including the 4-hydroxyl moiety [39]. However, we show here that
9 4*β*-dPE A does not need the oxygen in position 4 to display a strong HIV activating
10 effect (figure 1).
11 Our results show that the viral transcriptional effect of 4*β*-dPE A depends on the
12 activation of the novel PKCθ and the mitogen-activated protein kinase MEK (figure 5).
13 Previous literature showed the importance of PKCθ in T lymphocytes [33] and thus, the
14 PKCθ activation was further studied by immunofluorescence (figure 7). The effect of
15 phorbol esters on PKCθ and its HIV anti-latency activity have been widely described. On
16 the other hand, the effect of MEK inhibition in PKC activity has been previously
17 described [40], but less is known about its involvement in latency reactivation. MEK
18 proteins are a family of intracellular dual kinases that activates ERK/MAPK in response
19 to diverse stimuli such as IL-2 and phorbol esters. The activation of this pathway is
20 strongly involved in cell proliferation and activation of T-cells. Therefore, PKCθ/MEK
21 activation is required for the transcriptional, and thus, anti-latency activity of 4*β*-dPE A.
22 On the other hand, receptors down-regulation effect, and thus, anti-HIV activity, seems
23 to be independent of PCK/MEK (figure 6). This fact suggests a dual target of 4*β*-dPE A

in T cells, PKCθ/MEK as target for transcriptional activation and a still unknown target as an HIV infection inhibitor. Different chemical moieties could be involved in each target.

We have further explored the mechanism of transcriptional activation. 4β-dPE A induces a clear transactivation of NF-κB transcription factor (figure 8). HIV LTR contains binding sites for NF-κB and its activation is crucial for the initiation of transcription of latent HIV DNA. These effects are similar to those exerted by prostratin and SJ23B [32, 19] but 4β-dPE A is a powerful activating agent of HIV transcription, since similar concentrations than bryostatin and lower concentrations than the other phorbol esters are needed in all the scenarios tested (figure 3 and table 3). In fact, our results show that 4β-dPE A is also more powerful than HDACIs as vorinostat and thus, it could be used at lower doses in clinical assays.

It has been previously described that anti-latency treatment could be more efficacious with combinations of different drugs. However, in clinical assays only antibody-LRA combinations have been tested [20, 21] unlike drug/drug combinations. Combination of drugs with different targets would be, hypothetically, synergistic. This is the case of the combination of PKC agonists such as prostratin or 4β-dPE A with an HDAC inhibitor as vorinostat. In fact, our results show that these combinations are strongly synergistic, while the combinations of two PKC agonists such as 4β-dPE A and prostratin showed a strong antagonism. Therefore, the combination of LRAs with different targets would result in a synergism able to diminish the doses used to obtain enough activation to reduce the reservoir size. Moreover, the combination of 4β-dPE A and vorinostat was synergistic not only lowering EC_{50}s values but also increasing their transcriptional efficacy (figure 9). Thus, the combination of these two drugs would result in the

1 possibility of lowering doses and, at the same time, increasing the efficacy as viral
2 reservoir reactivators.
3 Since latency reversing therapy is proposed to be used together with ART in infected
4 patients, the effect of LRAs is a matter of concern if a negative effect results from this
5 treatment in two different aspects: the main one is the potential toxicity due to LRA
6 treatment on its own in patients with excellent viral control and quality of life. Besides, an
7 antagonistic effect of LRA with ART could be envisaged and as far as we know this
8 potential pharmacological interaction has not been assessed. In this article we have
9 analyzed the effect of 4*β*-dPE A on anti-HIV activity when combined with different
10 families of types of antiretroviral drugs. Our results show a consistent synergism as HIV
11 infection inhibitors of LRA with nucleoside or nucleotide reverse transcriptase inhibitors
12 (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors
13 (PIs) and even with the CCR5 antagonist maraviroc. This synergistic effect would be
14 expected for NRTIs, NNRTIs and PIs, since 4*β*-dPE A down-regulates CD4, CXCR4 and
15 CCR5 receptors, inhibiting HIV-1 entry and, thus, diminishing the rate of infection of T
16 cells. However, the down-regulation of CCR5 could theoretically interfere with the
17 maraviroc activity. We did not find antagonism between 4*β*-dPE A and maraviroc in our
18 experiments ruling out a potential antagonism (Figure 10). On the other hand,
19 antiretroviral drugs did not interfere with the transcriptional activity of PKC agonists PMA
20 and 4*β*-dPE A (figure 11), and thus, the efficacy of deoxyphorbols *in vivo* would not be
21 altered by the antiretroviral treatment.
22 Finally, the reservoirs of HIV in latently infected cells are thought to be long-lived resting
23 memory CD4+ T cells [41]. This reservoir is formed in the early phases of the infection
24 harboring integrated proviral DNA. Shock therapy tries to activate specifically these cells

to turn them visible to the immune system or to the kill therapy. Therefore, we have treated CD4+T cells from infected patients under ART with 4β-dPE A to evaluate the reactivation effect in latently infected cells. As we show in figure 13, 4β-dPE A reactivated HIV from CD4+ T cells from infected patients at a concentration of 100 nM and thus, 4β-dPE A could display a strong reactivating effect on the viral reservoir *in vivo*.

In summary, we show here that a 4-deoxyphorbol derivative, 4β-dPE A, inhibits HIV infection through CD4, CXCR4 and CCR5 down regulation and reactivates HIV in resting PBMCs through PKCθ/MEK and NF-κB activation. This last effect is independent of receptors downregulation, since inhibition of PKC/MEK does not modify receptor expression inhibition induced by 4β-dPE A, and, thus, other cellular targets should be involved. Moreover, 4β-dPE A shows a strong synergism with HDAC inhibitor vorinostat as LRA and with NRTIs, NNRTIs and PIs as HIV inhibitor. Finally, 4β-dPE A activity in CD4+ T cells from infected patients under ART will confirm its effect in a more real scenario. Therefore, 4β-dPE A is a new deoxyphorbol ester derivative with powerful activity as anti-latency agent and HIV infection inhibitor which could be a drug candidate to eradicate HIV reservoirs.

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6. CONFILCT OF INTERESTS

There are no conflicts of interest

7. FIGURE LEGENDS

Figure 1. Chemical structures of 4*β*-dPE A, phorbol myristate acetate (PMA) and prostratin. The hydroxyl group in position 4 of phorbol derivatives is not needed for its activity as a PKC agonist and a HIV-1 reactivating agent.

Figure 2. A: Anti-HIV-1 activity and toxicity of 4*β*-dPE A. A: MT-2 cells or IL-2 activated PBMCs were pretreated with different concentrations of 4*β*-dPE A and infected with an X4 or R5 tropic HIV or with a HIV-VSV virus to assess the receptors dependence of the antiviral activity. Luciferase-renilla activity (RLUs) in cell lysates was measured 48 h later. Results are expressed as % RLUs versus infection in the presence of the same concentration of DMSO used to dissolve the drug (100%). Long term toxicity of 4β-dPE A was evaluated in pre-activated PBMCs

treated with different concentrations of compound, and left in culture for two weeks. Every 48–72 h, cell culture was washed and treatment was added again. Cell viability was measured at days 2, 6, 9 and 14 by CellTiter Glo luminescence viability assay, and results are shown as percentage of viable cells compared to a non-treated culture (100%). Cell Viability was assessed with CellTiter Glo luminescence viability assay**. B: Anti-HIV-1 activity and toxicity of LRAs.** MT-2 cells or IL-2 activated PBMCs were pretreated with different concentrations of bryostatin-1, prostratin, PMA or vorinostat and infected with an X4 tropic HIV. Renilla activity (RLUs) in cell lysates was measured 48 h later. Results are expressed as % RLUs versus infection in the presence of the same concentration of DMSO used to dissolve the drug (100%). Cell Viability was assessed with CellTiter Glo luminescence viability assay**.**

Figure 3. A: HIV-1 transcriptional reactivation activity of 4*β*-dPE A. **A**: MT-2 cells and resting PBMCs were transfected with pNL4.3-Luc (HIV-1). TZMbl and Jurkat 5.1 LTR-Luc are cells lines stably transfected with an HIV-1 LTR-Luc and transcriptional activity can be evaluated directly by measuring the luciferase activity (%RLUs). MT-2 cells, TZMbl, Jurkat 5.1-LTR-Luc and resting PBMCs were then treated with either 4*β*-dPE A (1, 10 or 100 nM) or PMA (100 nM), and cultured at 37°C for 48h. RLUs were then determined in a luminometer. Results are expressed as % RLUs versus infection in the presence of the same concentration of DMSO used to dissolve the drug (100%). ANOVA analysis was performed to determine the value of p (* $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$). All the results are the mean of at least 3 different replicates. **B: Transcriptional activity of 4*β*-dPE A and LRAs.** Resting PBMCs were transfected with pNL4.3-Luc (HIV-1) and treated with serial dilutions of 4*β*-dPE A, prostratin, PMA. bryostatin, and vorinostat and cultured at 37°C for 48h. Results are expressed as % RLUs of treated cells versus untreated in the presence of the same concentration of DMSO used to dissolve the drug (100%).

1 **Figure 4. Effect of 4*β*-dPE A on CD4, CXCR4 and CCR5 receptors expression**. Upper
2 panels: Kinetics of expression of CD4 and CXCR4 receptors induced by 4*β*-dPE A in MT-2 cells.
3 Lower panels: Kinetics of expression of CD4, CXCR4 and CCR5 receptors induced by 4*β*-dPE A
4 in IL-2 activated PBMCs. Cells were treated with either 4*β*-dPE A at 1, 10 or 100 nM or PMA
5 (100 nM) and expression of membrane receptors was measured using specific monoclonal
6 antibodies at different points along drug treatment (2, 24 and 48 h). Results are expressed as
7 percentage of expression measured as mean intensity fluorescence (MIF) compared with
8 untreated cells (100%). All the results are the mean of at least 3 different replicates.

9 **Figure 5**. **PKC and MEK dependence of the HIV-1 transcriptional activity of 4*β*-dPE A**.
10 Resting PBMCs were transfected with pNL4.3-Luc (HIV-1) and maintained in cell culture. Cells
11 were then treated with either 4*β*-dPE A (10 or 100 nM) or PMA (100 nM), in the presence of
12 PKCs inhibitors bisindolylmaleimide 1 μM (pan-PKC inhibitor) or Gö6976 1 μM (classical PKC
13 inhibitor), MEK inhibitor 1 μM (IMEK) and specific PKCθ inhibitor rottlerin 3 μM and cultured at
14 37°C for 48h. 100% represents the background level of expression without treatment. Statistics
15 was performed using a t-test to determine the value of p (* p <0.05). All the results are the mean
16 of at least 3 different replicates.

17 **Figure 6. Kinetics of expression of CD4, CXCR4 and CCR5 receptors** induced by 4*β*-dPE A
18 in IL-2 activated PBMCs in the presence of: A) Pan PKC inhibitor bisindolylmaleimide (Bis 1 μM)
19 B) MEK inhibitor (IMEK 1 μM) C) Specific PKCθ inhibitor rottlerin (Rot 3 μM). Cells were treated
20 with either 4*β*-dPE A at 10 or 100 nM and membrane expression of receptors was measured
21 using specific monoclonal antibodies at different points along drug treatment (2, 24 and 48 h).
22 Results are expressed as percentage of expression measured as mean intensity fluorescence
23 (MIF) compared with 4*β*-dPE A untreated cells without PKC or MEK inhibitors (100%). All the
24 results are the mean of at least 3 different replicates.

Figure 7. Immunofluorescence PKCθ assay. PBMCs were treated with PMA (100 nM), 4*β*-dPE A (100 nM) or DMSO (vehicle). Then, cells were permeabilized with the cell fixative CytoskelFix for 10 min and incubated with phospho-PKCθ (Thr538) and Alexa Fluor 546 (red) goat anti-rabbit IgG antibodies. Dapi was used for nuclear staining. Images were obtained with Leica DMI 4000B Inverted Microscope. Intensity mean per pixel was calculated and values were represented in bar diagrams showing statistical significance. The results shown are a representative one of at least 3 different replicates.

Figure 8. Effect of 4*β*-dPE A on HIV-1 LTR and NF-κB and NFAT transcription factors. Resting PBMCs were transfected with plasmids encoding a luciferase reporter gene under the control of the HIV LTR or NF-κB or NFAT transcription factors. Afterwards cells were treated with either 4*β*-dPE A at 1, 10 or 100 nM or PMA at 100 nM, and cultured at 37°C during 48h. 100% represents the background level of expression without treatment. T-test was performed to determine the value of p (** p <0.01, *** p <0.001). All the results are the mean of at least 3 different replicates.

Figure 9. Transcriptional activity combination experiments. **A:** Resting PBMCs were transfected with a recombinant HIV-1 (NL4.3-Luc) and treated with serial dilutions of the drugs alone or the combinations. EC_{50}s values of the drug's alone were used to calculate the ration of combination and combination curves were then performed in parallel to drug alone curves. EC_{50}: Effective concentration 50% in reactivation assays. CI95%: Confidence interval 95%. Effective concentration 50% to effective concentration 50% combination ratio (EC_{50}/EC_{50}) was used in these experiments. Combination index values at EC_{50} were obtained using CalcuSyn software. Combination index > 1.30 = antagonism, 1.10–1.30 = weak antagonism, 0.90–1.10 = additive, 0.70–0.90 = weak synergy, less than 0.70 = strong synergy. Values are shown as % of RLUs compared to a non-treated control (100%). **B:** Maximum efficacy (Emax) comparisons were

1 performed with the top effects obtained for each combination. 100% is the value of the untreated
2 control. T test ** $p<0.01$. All the results are the mean of at least 3 different replicates.

3 **Figure 10. HIV inhibition combination experiments**. **A: Anti-HIV activity of the**
4 **antiretroviral drgus tested.** MT-2 cells or PBMCs were treated with different concentrations of
5 the antiretrovirals lamivudine (3TC), emtricitabine (FTC), abacavir, tenofovir, efavirenz, ritonavir
6 and maraviroc (MVC) and infected with HIV-1. After 48 hours, cell culture was lysed and RLUs
7 obtained in a luminometer. IC_{50} values were calculates using Graph Pad prism software. **B:**
8 **Evaluation of the anti-HIV-1 activity of the different drug combination tested**. $IC_{50}A/IC_{50}B$
9 ratio was used to combine the drugs in all the combinations tested. Drugs alone and
10 combination curves were performed in parallel to compare the effect of the combination against
11 drugs alone. Combination index values were obtained using CalcuSyn software at EC_{50}, EC_{75}
12 and EC_{90} (effective concentrations). Combination index > 1.30 = antagonism, 1.10–1.30 = weak
13 antagonism, 0.90–1.10 = additive, 0.70–0.90 = weak synergy, less than 0.70 = strong synergy.
14 Values are shown as % of RLUs compared to a non-treated control (100%). Toxicity of all the
15 compounds and combinations were tested in parallel. All the results are the mean of at least 3
16 different replicates.

17 **Figure 11. Viral reactivation combination experiments**. Resting PBMCs were transfected
18 with a recombinant HIV-1 (NL4.3-Luc) and treated with a single concentration of PMA or 4*β*-dPE
19 A (100 nM) to be tested or a combination with an antiretroviral abacavir (ABC 1 μM), efavirenz
20 (EFV 0.01 μM), ritonavir (Rito 0.1 μM), lamivudine (3TC 1 μM), emtricitabine (FTC 1 μM) and
21 tenofovir (TFV 1 μM). Values are shown as % of RLUs compared to a non-treated control
22 (100%). Toxicity of all the compounds and combinations were tested in parallel. Statistical
23 analysis was performed using the Graph Pad Prism software (T test). All the results are the
24 mean of at least 3 different replicates.

Figure 12. Transformation assay. NIH 3T3 fibroblasts were treated with prostratin, 4β-dPE A or PMA. All compounds were used at 0.1 μM concentration. As positive control, K-Ras V12 (pCEFL-KZ-HAK-Ras V12) was transfected by the calcium phosphate precipitation technique. Morphologically transformed foci were detected after 2–3 weeks in culture by giemsa staining of cell cultures. As negative control, vector pCEFL-KZ-HA was transfected under similar conditions. Transformant foci were counted using Image J software. The results shown are a representative one of at least 3 different replicates.

Figure 13. Anti-latency effect of 4β-dPE A in CD4 T cells of ART treated patients. CD4+ T cells from PBMCs obtained from infected patients under ART were isolated by positive selection using CD4+microbeads. Cells were then cultured in the presence or not of 100 nM concentration of 4β-dPE A, PMA or left untreated as control. After 48 hours, culture supernatants were collected and submitted to viral load quantitation. Results are obtained from samples from 3 different patients. A two-way ANOVA analysis were then performed using Graph Pada Prism software.

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