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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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4 **4-deoxyphorbol inhibits HIV-1 infection and**
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11 **activation synergizing with vorinostat and**
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15 **antiretroviral drugs**
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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 agonists of PKCs such PMA and prostratin and to diterpene
9 esters such SJ23B. 4 β -dPE A lacks the long side chain present in PMA, suggesting a
10 non-tumour promoting activity, as our results indicate. 4 β -dPE activates HIV
11 transcription at nanomolar concentrations, lower than the concentration needed other
12 latency reversing agents (LRAs) such as prostratin or bryostatin. PKC θ /MEK activation
13 is required for the transcriptional activity, and thus, anti-latency activity of 4 β -dPE A.
14 However, CD4, CXCR4 and CCR5 receptors down-regulation effect seems to be
15 independent of PCK/MEK, suggesting the existence of at least two different targets for
16 4 β -dPE A. Further, NF- κ b transcription factor is involved in 4 β -dPE HIV reactivation, as
17 previously shown for other PKCs agonists. We also studied the effects of 4 β -dPE A in
18 combination with other LRAs. When 4 β -dPE A was combined with another PKC
19 agonists such as prostratin an antagonist effect was achieved, while, when combined
20 with an HDAC inhibitor such as vorinostat, a strong synergistic effect was obtained.
21 Interestingly, the latency reversing effect of the combination was synergistic diminishing
22 the EC₅₀ value but also increasing the efficacy showed by the drugs alone. Finally,
23 combination of 4 β -dPE A with antiretroviral drugs as CCR5 antagonist, NRTIs, NNRTIs

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1 and PIs, showed a consistent synergistic effect, suggesting that the combination would
2 not induce undesirable effects on antiretroviral therapy (ART). In summary, we describe
3 here the mechanism of action of a new potent deoxyphorbol derivative as a latency
4 reversing agent candidate to decrease the size of HIV reservoirs.

5 **Keywords:** HIV-1, latency reversing agents, proteinkinases C, viral reactivation, phorbol
6 esters

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1 **1. INTRODUCTION**

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

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

18 The latency-reversing agents (LRAs) or anti-latency drugs will be the "shock" of this
19 strategy. There are several kinds of LRAs including, epigenetic modifiers, TLR agonists,
20 TCR activators, PI3K/Akt pathway modulators, NF-κB agonists, PKC agonists and
21 HDAC inhibitors [8]. PKCs agonists (prostratin or bryostatin) and HDAC inhibitors
22 (vorinostat, panobinostat or romidepsin) are the most studied LRAs. Moreover, some

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

16 PKCs agonist can activate some isoforms of protein kinases C (PKCs) by binding to its
17 regulatory C1 domain, mimicking the action of the physiological ligand diacylglycerol
18 (DAG) and inducing viral reactivation. Prostratin was the first PKC agonist **studied as a**
19 **potential anti-latency agent [18].** Bryostatin, **another PKC agonist**, showed lack of
20 efficacy in the reduction of the latent reservoir **in clinical trials**, probably due to the low
21 doses used in the assay to avoid toxicity since systemic concentrations were found
22 undetectable [10, 19]. In this sense, some diterpenes and phorboids from
23 *Euphorbiaceae* have also been described as LRAs with antiviral properties and

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1 reactivation activity. However, slight differences in their structure lead to lack of activity
2 or a PKC isoform preference, modulating their reservoir reactivating activity [20].
3 Identification and characterization of new phorboids with different PKCs binding profile,
4 identification of other potential targets of these drugs and selection of non-tumorigenic
5 phorboids with low toxicity could be helpful for the treatment of HIV-1 latency and the
6 cure of infection.

7 Finally, as in ART, reactivation of the latent reservoir **could only be** achieved by the
8 combination of different drugs or antibodies able to diminish doses, and thus toxicity,
9 and to achieve cell activation enough to eliminate the reservoirs [21, 22]. In this sense, it
10 is important to point out that this therapy is intended to be used in combination with
11 ART. Some previous reports have shown that antiretroviral drugs could modulate the
12 LRAs activity and, conversely, LRAs can affect the antiviral activity of ART. For example,
13 some protease inhibitors diminish the viral reactivation obtained with LRAs [23].

14 We have reported previously the antiviral activity of 4-deoxyphorbol derivative
15 compounds isolated from *Euphorbia amygdaloides* ssp. *semiperfoliata* [24]. In the
16 present study, the mechanism of action and cellular pathways involved in the latency-
17 reversal and anti-HIV-1 effects of 4 β -dPE A, have been evaluated. This compound
18 shows antiviral activity through down-regulation of receptors and is able to induce HIV
19 reactivation. This class of compounds could be used as an adjuvant therapy to eliminate
20 the latent reservoirs of HIV-1.

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22 **2. MATERIAL AND METHODS**

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1 **Reagents**

2 *4β*-dPE A was isolated from *Euphorbia amygdaloides* ssp. *semiperfoliata* and provided
3 by CNRS (Centre National de la Recherche Scientifique CNRS, France). The compound
4 was solubilized in DMSO to a final concentration of 10 mM and stored aliquoted at -
5 80°C. Monoclonal antibodies (mAbs) to CD4, CXCR4 and CCR5 were supplied by
6 Becton Dickinson (Mountain View, CA, USA). Interleukin-2 (IL-2) was supplied by Chiron
7 (Emeryville, CA, USA). The inhibitor rottlerin was obtained from Alexis Co. (Lausanne,
8 Switzerland) and the inhibitors Gö6850 and Gö6976 were obtained from Calbiochem
9 (EMD Biosciences, Inc. Darmstadt, Germany). Renilla and Luciferase Assay Systems
10 and CellTiter Glo viability assay were supplied by Promega (Madison, WI, USA). DMSO
11 and MEK1/2 inhibitor PD184352 were supplied by Sigma-Aldrich (St Quentin-Fallavier,
12 France). The following reagents were obtained through the NIH AIDS Reagent Program,
13 Division of AIDS, NIAID, NIH: SAHA (vorinostat), raltegravir, lamivudine, tenofovir,
14 emtricitabine, abacavir, efavirenz and ritonavir. Romidepsin and panobinostat were
15 purchased from Selleck Chemicals (Houston, TX) and prostratin were purchased from
16 Sigma-Aldrich (St Louis, MO).

17 **Cells**

18 MT-2 cells (American Type Culture Collection, Manassas, VA, USA, Ref: CRL-2560)
19 were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 2 mM L-
20 glutamine, penicillin (50 IU/ml) and streptomycin (50 mg/ml) (all Whittaker M.A. Bio-
21 Products, Walkerville, MD, USA) and split twice a week. 293T cells, obtained from the
22 American type culture collection ATCC ref: ATCC® CRL-3216™, and TZM-bl cells (cell
23 line stably transfected with an LTR-Luc), obtained through the NIH AIDS Reagent
24 Program, Division of AIDS, NIAID, NIH: TZM-bl cells (Cat#8129) from Dr. John C.

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1 Kappes, and Dr. Xiaoyun Wu, were both cultured in DMEM medium containing 10%
2 (v/v) fetal bovine serum, 2 mM L-glutamine, penicillin (50 IU/ml) and streptomycin (50
3 mg/ml) (Whittaker) and split twice a week. PBMCs (peripheral blood mononuclear cells)
4 were isolated from healthy blood donors by centrifugation through a Ficoll-Hypaque
5 gradient (Pharmacia Corporation, North Peapack, NJ) and were suspended in RPMI
6 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and
7 antibiotics (100 mg/ml streptomycin and 100 U/ml penicillin) (all Whittaker M.A. Bio-
8 Products, Walkerville, MD, USA) before culture at a concentration of 2×10^6 cells/mL. In
9 some experiments, PBMCs were activated with IL-2 (300 IU/mL) for at least 48 h. Cells
10 were cultured at 37 °C in a 5% CO₂ humidified atmosphere. Proper informed consent
11 was obtained from each subject in accordance with the Spanish legislation on blood
12 donor regulations. Confidentiality and privacy was assured.

13 **Plasmids**

14 The vector pNL4.3-luc was generated by cloning the luciferase gene in the HIV-1
15 proviral clone pNL4.3 [25]. Plasmid pNL4.3-Ren was generated cloning the renilla gene
16 in the *nef* site of pNL4.3 and pJR-Ren plasmid was generated cloning the *env* gene of
17 HIV-1 JR_{CSF} in the pNL4.3-Ren plasmid [26]. pNL4.3-Δenv-Luc was obtained through
18 the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3.Luc.R⁻.E⁻ from
19 Dr. Nathaniel Landau and pcDNA-VSV encoding the G protein of VSV from Dr.
20 Arenzana-Seisdedos from Pasteur Institute [27]. The 3-enh-κB-ConA-luc plasmid carries
21 a luciferase gene under the control of three synthetic copies of the κB consensus of the
22 immunoglobulin k-chain promoter cloned into the BamHI site located upstream from the
23 conalbumin transcription start site [28]. The LTR-Luc plasmid carries a luciferase gene

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1 under the control of HIV-1- LTR region [29]. The expression construct pNFAT-LUC
2 containing three tandem copies of the distal NFAT-binding site of the IL-2 gene promoter
3 coupled to the IL-2 minimal promoter was kindly provided by Dr. Juan Miguel Redondo
4 (National Center for Cardiovascular Research, Madrid, Spain) [30].

5 Plasmids were amplified transforming *E. coli* DH5 α cells and cultured in LB
6 supplemented with ampicillin at 37°C for 18 hours. All plasmids were purified using
7 Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) following the manufacturer
8 instructions. DNA concentration was determined in a Nanodrop (Thermoscientific,
9 Waltham, MA, USA).

10 **Viruses**

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

17 **Anti-HIV-1 activity evaluation by recombinant virus assay**

18 The initial evaluation of 4 β -dPE A was performed in MT-2 cells infected with NL4.3-Ren
19 obtained previously by transfection in 293T cells. The assay was performed in 96 well
20 microplate seeded with 100,000 cells/well in a final volume of 200 μ l. Cell cultures were
21 infected with viral supernatants (100,000 RLU/w) and after 48 hours cell pellets were
22 lysed and luciferase activity measured in a luminometre (Berthold detection systems,

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1 Pforzheim, Germany). X4 (NL4.3-Ren) or R5 (JR-Ren) infections of IL-2 activated
2 PBMCs followed the same procedure. All the experiments were controlled with cells
3 treated with the same DMSO concentration of 4 β -dPE A. HIV-1 replication inhibition was
4 evaluated by measuring the luminescence activity or RLUs, with 100% being the
5 infection of non-treated cells. IC₅₀ was calculated with a non-linear regression formula
6 using GraphPad Prism software.

7 **Toxicity evaluation**

8 Cell toxicity was evaluated by treating mock infected cells with the same concentrations
9 of 4 β -dPE A used in the antiviral assays or with higher concentrations of 4 β -dPE A and
10 other LRAs (panobinostat, romidepsin and prostratin) or its combination with LRAs or
11 antiretroviral drugs. After 48 hours in culture, CellTiter Glo reagent (Promega, Madison,
12 WI, USA) was added and cell viability measured by RLUs quantification in a
13 luminometre. Cell viability is expressed as percentage of viable cells compared to a non-
14 treated (DMSO same concentration as compound) control (100%). Long-term toxicity
15 evaluation at different times was performed with 4 β -dPE A in IL-2-activated-PBMCs for
16 14 days. CC₅₀ was calculated with a non-linear regression formula using GraphPad
17 Prism software.

18 **Receptor expression kinetics**

19 Single-, double- or three-color immunophenotyping was performed with a FACScalibur
20 flow cytometer using CD4 (clone SK3), CXCR4 (clone 12G5) and CCR5 (clone
21 2D7/CCR5) antibodies conjugated to the appropriate fluorochrome, all obtained from
22 Becton Dickinson (Heidelberg, Germany). Background staining was assessed with the

1 appropriate isotype- and fluorochrome-matched control mAb and subtracted. Activated
2 PBMCs or MT-2 were treated with different concentrations of 4 β -dPE A for 48, 24 and 2
3 hours. Cells were then collected and CD4, CXCR4 and/or CCR5 receptors expression
4 were evaluated. The effect of PKCs inhibitors **Gö6850 (bisindolylmaleimide)**, rottlerin
5 and MEK inhibitor PD184352 was also studied in the expression of CD4, CXCR4 and
6 CCR5 receptors in IL-2 activated PBMCs. The analysis was performed using forward-
7 versus-side scatter dot plots. Results are shown as percentage of receptor fluorescence
8 mean intensity (%MIF) obtained by FlowJo 8.0 software.

9 **Immunofluorescence Assays**

10 For immunofluorescence assays, Jurkatt E61 cells and PBMCs were incubated in a μ -
11 Slide 8 well ibiTreat (Ibidi GmbH) for 48h, in the presence or absence of PMA or 4 β -dPE
12 A (100 nM). Then, they were permeabilized with the cell fixative CytoskelFix
13 (Cytoskeleton) for 10 min at room temperature. After washing twice with 0.1%
14 glycine/PBS, cells were incubated with phospho-PKC θ (Thr538) (Cell Signaling
15 Technology, Danvers, MA) and Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes)
16 antibodies. Washes were performed with 1X PBS–2% BSA–0.05% saponine buffer. 4',6-
17 diamidino-2-phenylindole (Dapi) was used for nuclear staining. Images were obtained
18 with Leica DMI 4000B Inverted Microscope (Leica Microsystems, Wetzlar, Germany).

19 **Evaluation of viral transcription**

20 MT-2 cells or resting PBMCs (in culture for at least 24 hours) were suspended in 350 μ L
21 of RPMI without supplements and electroporated using an Easyject plus Electroporator
22 (Equibio, Middlesex, UK) at 260 (MT-2) or 320 (PBMCs) V, 1500 mF and maximum

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1 resistance with $1\mu\text{g}/10^6$ cells of a luciferase plasmid under the control of the whole
2 genome of HIV-1 (NL4.3-Luc). Afterwards MT-2, resting PBMCs or TZM-bl (non-
3 transfected) cells were seeded in 24 well microplates and treated with different
4 concentrations of 4β -dPE A or PMA ($0.1\ \mu\text{M}$), as a reference control of HIV-1
5 transactivation, and left in culture in complete RPMI at 37°C . 48 hours later, cultures
6 were lysed with luciferase buffer and RLUs measured in a luminometer. This procedure
7 was also used for the transfection of luciferase plasmids under the control of
8 transcription factors NFAT (NFAT-Luc) or NF- κ B (3-enh- κ B-ConA-luc) or the long
9 terminal repeat of HIV (LTR-Luc) in resting PBMCs to evaluate transcriptional activity.
10 The transcriptional activity of 4β -dPE A in resting PBMCs was also studied in the
11 presence of PKCs inhibitors Gö6850 (Bisindolylmaleimide), Gö6976 or rottlerin and
12 MEK inhibitor PD184352.

Combination experiments

14 4β -dPE A was tested as a latency reversing agent alone and in combination with other
15 latency reversing agents as prostratin and vorinostat and a single concentration of
16 antiretrovirals abacavir (ABC $1\ \mu\text{M}$), efavirenz (EFV $0.01\ \mu\text{M}$), ritonavir (Rito $0.1\ \mu\text{M}$),
17 lamivudine (3TC $1\ \mu\text{M}$), emtricitabine (FTC $1\ \mu\text{M}$) and tenofovir (TFV $1\ \mu\text{M}$). The model
18 used to evaluate the anti-latency effect was performed in primary PBMCs isolated from
19 healthy donors. Briefly, resting PBMCs were transfected with a recombinant HIV-1
20 (NL4.3-Luc) and treated with serial dilutions or single concentrations or the drugs to be
21 tested. After 48 hours, cell culture was lysed and RLUS were obtained in a luminometer.
22 A control of the vehicle used for the dilution of the drugs was used for all the
23 concentrations tested (100%). For LRAs, EC_{50} and maximum effect (Emax) were

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1 calculated for each drug separately and EC_{50}/EC_{50} ratios were determined. Afterwards,
2 combinations experiments were performed following the same procedure with the drugs
3 alone as compared to their EC_{50}/EC_{50} combination. For antiretrovirals, a T-Test analysis
4 were performed between the reactivation rates obtained with PMA or 4 β -dPE A alone
5 and the combination with the antiretroviral drug.

6 4 β -dPE A was also evaluated in combination with antiretrovirals such as the nucleoside
7 analogue reverse transcriptase inhibitors (NRTI) lamivudine, emtricitabine and abacavir,
8 the nucleotide analogue reverse transcriptase inhibitor (NtRTI) tenofovir, the non-
9 nucleoside analogue reverse transcriptase inhibitor (NNRTI) efavirenz and the protease
10 inhibitor (PI) ritonavir. Briefly, MT-2 cells were pre-treated with serial dilutions of the
11 drugs to be tested for 30 minutes and infected with a recombinant HIV-1 (NL4.3-Ren).
12 After 48 hours, cell culture was lysed and RLUS were obtained in a luminometer. A
13 control of the vehicle used for the dilution of the drugs was used for all the
14 concentrations tested (100%). IC_{50} was calculated for each drug separately and IC_{50}/IC_{50}
15 ratios were determined. Afterwards, combinations experiments were performed following
16 the same procedure with the drugs alone as compared to their IC_{50}/IC_{50} combination.

17 **Transformation assays**

18 NIH 3T3 fibroblasts were maintained in medium DMEM supplemented with 10% calf
19 serum and treated either with SJ23B, prostratin, or PMA. All compounds were used at
20 0.1 mM concentration. As positive control, K-Ras V12 (pCEFL-KZ-HAK-Ras V12) was
21 transfected by the calcium phosphate precipitation technique. As negative control,
22 vector pCEFL-KZ-HA was transfected under similar conditions. Morphologically
23 transformed foci were scored after 2–3 weeks in culture [31]. Cells were washed with

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1 PBS 1x, fixed with PFA 4% for 30 min, washed again with deionized water and stained
2 with Giemsa staining solution. Transformed cell foci appeared intense blue colored.

3 **Statistics**

4 FlowJo 8.0 software was used to analyze flow cytometry data and results are shown as
5 percentage of receptor fluorescence mean intensity (%MIF) as compared to a non-
6 treated control (100%). GraphPad Prism software was used to calculate effective
7 concentration 50%, inhibitory concentration 50%, cytotoxic concentration 50% (EC₅₀,
8 IC₅₀, CC₅₀) and Emax (top) in all the experiments (Non-linear regression, dose-response
9 stimulation, log[agonist] vs response for anti-latency experiments and dose response
10 inhibition, log[inh] vs response for antiviral experiments) and to represent concentration-
11 response curves. Confidence intervals 95% (CI95%) and R² are provided for all the
12 calculated concentrations. GraphPad Prism was also used to perform ANOVA or T-test
13 analysis to determine the value of p (* p <0.05, ** p <0.01 *** p <0.001). Finally, the
14 Calcosyn Software was used to evaluate combination index (CI) for each combination.
15 All the experiments were performed at least in triplicates in three independent
16 experiments.

17
18 **RESULTS**

19 **4β-dPE A inhibits *in vitro* HIV infection with low cytotoxicity**
20 4β-dPE A is a phorbol derivative with special chemical characteristics, as the lack of the
21 oxygen at position 4 and thus, we aimed to study its potential antiviral and latency
22 reversing activity. To that end, first we used MT-2 cells infected with an X4 tropic
23 recombinant virus (NL4.3-Ren) in the presence of different concentrations of 4β-dPE A.

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1 A strong inhibition of viral replication was observed at nanomolar concentrations, with an
2 IC₅₀ value of 6.51 nM. Further, when IL-2 activated PBMCs were infected under the
3 same conditions with X4 or R5 tropic HIV, a stronger inhibition of viral replication was
4 obtained, with IC₅₀ values of 0.31 and 0.24 nM, respectively (Figure 1).

5
6 Cell death was not observed at the concentrations tested in both, the infection assay
7 and the cytotoxicity assay. Moreover, 4β-dPE A CC₅₀ was higher than 10,000 nM (10
8 μM) in PBMCs and MT-2 cells (Figure 1). While 4β-dPE A, prostratin and vorinostat
9 showed no cell toxicity in MT-2 cells and PBMCs at 10.000 nM, a similar pattern of cell
10 toxicity were found for romidepsin and panobinostat, although panobinostat only showed
11 PBMCs toxicity at concentrations close to 10.000 nM. Since toxicity is a cause of
12 concern for anti-latency drugs, we further evaluated 4β-dPE A toxicity in a long-term
13 viability assay (14 days) in IL-2 activated PBMCs at concentrations of 0.1, 1 and 10 μM.
14 Cell viability was determined at different times along treatment. As observed in figure 1,
15 4β-dPE A was not toxic to human PBMCs even at 14 days of treatment.

16
17 **4β-dPE A decreases the expression of CD4, CXCR4 and CCR5 receptors**
18 Viral entry involves the interaction of HIV gp120 and gp41 with CD4, CXCR4 and/or
19 CCR5 receptors. It has been described previously the down regulation of these
20 receptors by PKCs agonists such as prostratin [32] and SJ23B [20]. Therefore,
21 expression of cell surface receptors was measured by flow cytometry in MT-2 cells and
22 IL-2 activated PBMCs. Cell cultures were stimulated with PMA or 4β-dPE A and left in
23 culture for 2, 24 and 48 h (Figure 2).

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

6 **4 β -dPE A induces HIV transcription**

7 The very low number of latently HIV-infected cells *in vivo* makes purification and
8 biochemical analysis of these cells hard to achieve. Moreover, several studies show that
9 *in vitro* assays used to quantify the reservoir from PBMCs display a large range
10 depending on the technique used [33]. In this work, we have used a model based on
11 MT-2 cells and primary human resting PBMCs transfected with luciferase plasmids
12 under the control of HIV-1 whole genome (NL4.3-Luc). We have also tested
13 transcriptional activation in non-infected TZMbl cells, since this cell line contains in its
14 genome integrated copies of the luciferase gene under the control of the HIV-1 promoter
15 LTR.

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17 4 β -dPE A activates HIV transcription in all the scenarios tested, inducing high levels of
18 HIV-1 reactivation with low concentrations (Figure 3).

19
20 **PKC theta/MEK pathway is required for 4 β -dPE A HIV transcriptional activity**

21 To assess the PKC/MEK dependence of 4 β -dPE A-mediated antagonism of HIV latency,
22 resting PBMCs were treated with PKCs chemical inhibitors Gö6976 1 μ M (classical
23 PKCs inhibitor), bisindolylmaleimide 1 μ M (Gö6850, classical and novel PKCs inhibitor),

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4 1 rottlerin 3 μM (PKC θ theta inhibitor) or MEK inhibitor PD184352 (IMEK) 1 μM and HIV
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7 2 transcriptional activity of 4 β -dPE A was also evaluated (Figure 4).
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11 4 Surprisingly, novel PKC inhibition did not decreased HIV transcription induced by 4 β -
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14 5 dPE A in a statistically significant manner. However, a tendency could be detected
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16 6 ($p=0,1531$). However, PKC theta (rottlerin) and MEK inhibition decreased transcriptional
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19 7 reactivation induced by 4 β -dPE A and PMA, suggesting a strong PKC theta/MEK
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21 8 dependence.
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25 26 10 **PKC theta/MEK pathway is not involved in 4 β -dPE A activity on receptors down-** 27 28 29 11 **regulation**

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31 12 To study the specific PKC isoform involved in 4 β -dPE A receptor down -regulation, we
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34 13 treated IL-2 activated PBMCs with PKCs chemical inhibitors, bisindolyImaleimide or
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36 14 rottlerin, and we evaluated the 4 β -dPE A effect on CD4, CXCR4 and CCR5 receptor
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39 15 expression. Downstream PKCs signaling in T cells may involve Raf-Mek dependent
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41 16 activation of MAPKs, and thus MEK inhibition could also play a role in the 4 β -dPE A
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43 17 effect. Therefore, PBMCs were treated with MEK inhibitor PD184352 and the effect of
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46 18 4 β -dPE A in the surface cell receptor expression was also measured.
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51 20 Surprisingly, inhibition of PKCs or MEK did not modify the expression of CD4, CXCR4 or
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53 21 CCR5 receptors with or without 4 β -dPE A treatment, suggesting that PKC/MEK pathway
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56 22 is not related to the down-regulation of receptors exerted by 4 β -dPE A, and thus, to the
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58 23 anti-HIV activity. Further, the differences of receptors expression between PKC inhibitor
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1 treated cells, MEK inhibitor treated cells and cell treated with no inhibitor were not
2 significant, suggesting that this inhibitors did not affect the receptor expression by
3 themselves (Figure 5).

4
5 **4β-dPE A induces a different pattern of intracellular distribution of PKCθ**

6 Figure 6 shows the results of PKCθ translocation in response to PMA or 4β-dPE A
7 treatment. PKCθ is the putative target of PKCs agonist in T cells [34], and we have
8 performed this experiments in PBMCs and Jurkat E61 cells by immunofluorescence.

9
10 Results show a different pattern of intracellular distribution of PKCθ when PMA (used as
11 pan PKC agonist control) or 4β-dPE A are used. This results suggest that PKCθ is a
12 target of 4β-dPE A.

13
14 **NF-κB transcription factor is involved in 4β-dPE A transcriptional activity**

15 To further analyze the mechanism of 4β-dPE A reactivation activity, luciferase
16 expression vectors under the control of three tandem κB consensus repeats (NF-κB
17 Luc), NFAT (NFAT Luc) or LTR region of HIV (LTR Luc) were transfected in resting
18 PBMCs, treated with 4β-dPE A at three different concentrations (1, 10 and 100 nM) or
19 with PMA (100 nM) and luciferase activity was measured after 48 h in culture.

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22 As observed in figure 7, 4β-dPE A induced the activation of the HIV LTR and NF-κB
23 transcription factor with the highest concentration of 100 nM as PMA. However, NFAT

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4 1 was not influenced by 4 β -dPE A treatment while PMA 100 nM plus Ionomycine (Io)
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6 2 strongly increased its expression.
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11 4 **4 β -dPE A displays a synergistic effect when combined with vorinostat but not**
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14 5 **with prostratin**

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16 6 Since 4 β -dPE A is a PKC agonist, we tested its combination with another PKC agonist,
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18 7 prostratin, and with a histone deacetylase inhibitor (HDACi), vorinostat. First, we studied
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20 8 the anti-latency effect of each drug separately. As it can be seen in figure 8, all the three
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22 9 compounds showed viral reactivation in resting PBMCs. Combinations were evaluated
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24 10 among all the three drugs, including 4 β -dPE A/prostratin, 4 β -dPE A/vorinostat and
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26 11 prostratin/vorinostat and the combination effect on EC₅₀ and Emax was studied.
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33 13 4 β -dPE A displayed an EC₅₀ value of around 10 nM as transcriptional reactivator, which
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35 14 is at least 70 and 480 fold lower than the EC₅₀ of vorinostat and prostratin, respectively,
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37 15 although it was less potent than PMA (data not show, EC₅₀ 0.2 nM). The combination of
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39 16 4 β -dPE A with prostratin was antagonistic showing a clear tendency toward lowering of
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41 17 Emax value. Moreover, the combination index (CI) were above 16, showing a strong
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43 18 antagonism. On the other hand, combination of 4 β -dPE A with vorinostat showed a
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45 19 strong synergism, with a CI value of 0.20 as well as the combination of prostratin with
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47 20 vorinostat with a CI of 0.21 (Figure 8).
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55 22 **Antiretroviral drugs did not modify the reactivation capacity of 4 β -dPE A**

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57 23 To test whether antiretroviral drugs could have an effect on the reactivation capacity of
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59 24 4 β -dPE A, a single concentration combination experiment was performed (figure 9).
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1 Concentrations were selected as follows: we have used the EC₉₀ obtained from
2 reactivation experiments of 4β-dPE A and PMA and the IC₉₀ obtained from infection
3 experiments of antiretroviral drugs, since we found no reactivation effect of antiretroviral
4 drugs alone.

6 There were no significant differences among the rate of reactivation of PMA or 4β-dPE A
7 alone and the rate of reactivation of the combination with an antiretroviral drug. Only
8 abacavir showed a slight tendency to increase the viral reactivation exerted by PMA and
9 4β-dPE A, but it was not significant.

11 **4β-dPE A displays a strong synergistic antiviral effect in combination with NRTIs,**
12 **NtRTIs, NNRTIs and PIs**

13 Since the LRA treatment is designed to be administered together with ART, we
14 evaluated the effect of 4β-dPE A in the antiviral effect of antiretroviral therapy.
15 Therefore, combination experiments with different types of antiretroviral drugs were
16 performed. To that end, the entry inhibitor maraviroc (MVC), the nucleoside analogue
17 reverse transcriptase inhibitors (NRTI) lamivudine, emtricitabine and abacavir, the
18 nucleotide analogue reverse transcriptase inhibitors (NtRTI) tenofovir, the non-
19 nucleoside analogue reverse transcriptase inhibitor (NNRTI) efavirenz and the protease
20 inhibitor (PI) ritonavir were combined with 4β-dPE A using IC₅₀/IC₅₀ ratios (Figure 10).

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1 We have found that the anti-HIV effect of all the antiretroviral drugs tested in the
2 presence of 4 β -dPE A showed a strong synergism with CIs below 0.70 for all the
3 concentrations tested. However, the combination 4 β -dPE A/efavirenz showed only a
4 weak synergism with a CI of 0.78 and the combination with 4 β -dPE A/maraviroc was
5 less synergic when concentrations were higher (ED90) (Figure 10).

7 **4 β -dPE A do not induce cell transformation**

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

16 We found that both 4 β -dPE A and prostratin were unable to elicit transforming foci, while
17 transfection with KrasV12 strongly induced cell transformation. Further, PMA treatment
18 induced foci formation but to a lesser extent than KrasV12 (Fig. 11).

21 **DISCUSSION**

22 The identification of potent natural or synthetic PKC agonists lacking tumor-promoting
23 activity and cellular proliferative activities has opened new research avenues for the

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1 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 and could potentially contribute to decrease the viral reservoir size.

5 4 β -dPE A showed two effects in the HIV replication cycle. First, it inhibits HIV infection, reported previously by us [24] and secondly it induces HIV transcriptional activation. These effects have been described for other PKCs agonists such as phorbol 12-myristate 13-acetate (PMA), bryostatin [35], prostratin [32] or SJ23B [20]. However, PMA, a phorbol diester, induced activation of HIV-1, but its potent tumor promoting activity turns its therapeutic use unacceptable [36]. Prostratin, bryostatin and SJ23B are PKCs agonists, analogs of diacylglycerol (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 has been hypothesized that the antitumor activity of PKCs agonists in general could be related to a high PKC activation leading to its degradation by the proteasome, while the natural ligand DAG or phorbol esters as PMA, with long side chains, will be inactivated before PKC degradation, promoting cellular growth. Moreover, it has been shown that phorbol esters with long acyl chain(s) such as the PMA, induced a distinct intracellular translocation pattern of PKCs and that this activity rapidly decreased with a shortening of the acyl side chain [37].

21 Thus, just like prostratin, the 4 β -dPE A, lacking the long side chain, should not display this tumor promoting activity. Short and long term toxicity experiments *in vitro* support this fact. 4 β -dPE A CC₅₀ was not reached at least at 20 μ M in PBMCs and 50 μ M in MT-

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1 2 cells, displaying a high specificity as HIV inhibitors with a specificity index greater than
2 10000 for MT-2 cells and PBMCs. Other LRAs as panobinostat or romidepsin induced
3 cell toxicity at much lower concentrations. Moreover, we did not find long term toxicity
4 after a 2 weeks treatment of PBMCs (figure 1) and transformation assays confirm the
5 lack of tumor-promoting activity (figure 11).

6 Among PKCs agonists, only prostratin and bryostatin have entered clinical trials as HIV
7 anti-latency agents although results were disappointing. The reasons of this failure are
8 not clear, but prostratin is less powerful than other phorboids [20], including 4 β -dPE A,
9 and thus, doses used *in vivo* should be higher leading to toxic effects more easily. Later,
10 clinical assays with bryostatin were designed with the lowest doses possible to avoid the
11 potential toxicity of PKC agonists. However, clinical assays did not show any effect on
12 the transcription of latent HIV, due probably, as the authors stated, to low plasma
13 concentrations [10].

14 In this paper, we show that 4 β -dPE A is a powerful HIV reactivation agent, with *in vitro*
15 active concentrations in the low nM range in resting PBMCs. Indeed, 100 nM
16 concentration in both cell lines was enough to induce HIV transcription at higher levels
17 than PMA at the same concentration. In PBMCs resting, just 10 nM was enough to
18 reach the same level of HIV reactivation than PMA at 100nM. Moreover, 4 β -dPE A was
19 also at least 20 times more powerful than prostratin as an HIV infection inhibitor [24] and
20 this anti-HIV activity is due to the down-regulation of CD4, CXCR4 and CCR5, as
21 expected for a PKC agonist. These data showed that 4 β -dPE A is at least as effective as
22 SJ23B in receptor down-regulation and more effective than prostratin.

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1 On the other hand, 4 β -dPE A was also more powerful as transcriptional reactivator than
2 vorinostat and prostratin with EC₅₀ values 70 and 480 fold lower, respectively,
3 confirming the high potency showed previously in the infectious experiments and its high
4 specificity also as LRA since its specificity index is greater than 1000 (Figure 8). The
5 classical PKCs (cPKCs) and novel PKCs (nPKCs) have two tandem C1 domains in their
6 N-terminal domain, the C1a and C1b domains, which show high binding affinities for
7 DAG, phorbol esters and other PKC activators. Interestingly, phorbol esters binding sites
8 in PKC C1 domain have been described and chemical pharmacophores identified.
9 These pharmacophores included a triad of oxygenation including the 4-hydroxyl moiety
10 [38]. However, we show here that 4 β -dPE A does not need the oxygen in position 4 to
11 display a strong PKC activating effect (figure 12).

12
13 Our results shown that the viral transcriptional effect of 4 β -dPE A depends on the
14 activation of the novel PKC θ and the mitogen-activated protein kinase kinase MEK
15 (figure 4). Previous literature showed the importance of PKC θ in T lymphocytes [34] and
16 thus, the PKC θ activation was further confirmed by immunofluorescence (figure 6). The
17 effect of phorbol esters on PKC θ and its HIV anti-latency activity have been widely
18 described. On the other hand, the effect of MEK inhibition in PKC activity has been
19 previously described [39], but less is known about its involvement in latency reactivation.
20 MEK proteins are a family of intracellular dual kinases that activates ERK/MAPK in
21 response to diverse stimuli such as IL-2 and phorbol esters. The activation of this
22 pathway is strongly involved in cell proliferation and activation of T-cells. Therefore,
23 PKC θ /MEK activation is required for the transcriptional, and thus, anti-latency activity of

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1 4β-dPE A. On the other hand, receptors down-regulation, and thus, anti-HIV activity,
2 seems to be independent of PCK/MEK (figure 5). This fact suggests a dual target of 4β-
3 dPE A in T cells, PKCθ/MEK as target for transcriptional activation (anti-latency effect)
4 and a still unknown target as an HIV infection inhibitor. Different chemical moieties could
5 be involved in each target.

6 We have further explored the mechanism of transcriptional activation. 4β-dPE A induces
7 a clear transactivation of NF-κB transcription factor (figure 7). HIV LTR contains binding
8 sites for NF-κB and its activation is crucial for the initiation of transcription of latent HIV
9 DNA. These effects are similar to those exerted by prostratin and SJ23B, the viral
10 transcriptional activation through the NF-κB transcription factor [32, 20]. **Therefore,**
11 **phorbol esters act through the activation of HIV-1 gene transcription, although 4β-dPE A**
12 **seems to be a more powerful activating agent of HIV transcription, since lower**
13 **concentrations than the other phorbol esters are needed in the three cell lines tested**
14 **(SJ23B EC₅₀ between 4-8 nM; prostratin EC₅₀ between 2000-3000 nM, data not shown),**
15 and thus, it could be used at lower doses in clinical assays. **In fact our results show that**
16 **4β-dPE A is also more powerful than HDACIs, since vorinostat showed an EC₅₀ value of**
17 **80-120 nM. Panobinostat and romidepsin were too toxic in our assays in both PBMCs**
18 **and cell lines, and did not allow EC₅₀ calculation (figure 1).**

19 Finally, it has been previously described that anti-latency treatment could be more
20 efficacious with combinations of different drugs. However, in clinical assays only
21 antibody-LRA combinations have been tested [21, 22] unlike drug/drug combinations.
22 Combination of drugs with different targets would be, hypothetically, synergistic. This is
23 the case of the combination of PKCs agonists such as prostratin or 4β-dPE A with an

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1 HDAC inhibitor as vorinostat. In fact, our results show that these combinations are
2 strongly synergistic, while the combinations of two PKCs agonists such as 4 β -dPE A
3 and prostratin showed a strong antagonism. Therefore, the combination of LRAs with
4 different targets would result in a synergism able to diminish the doses used to obtain
5 enough activation to reduce the reservoir size. Moreover, the combination of 4 β -dPE A
6 and vorinostat was synergistic not only lowering EC₅₀s values but also increasing the
7 Emax (figure 8). Thus, the combination of these two drugs would result in the possibility
8 of lowering doses and, at the same time, increasing the efficacy as viral reservoir
9 reactivators.

10 Since latency reversing therapy is proposed to be used together with ART in infected
11 patients, the effect of LRAs is a matter of concern if a negative effect results from this
12 treatment in two different aspects: the main one is the potential toxicity due to LRA
13 treatment on its own in patients with excellent viral control and quality of life. Besides, an
14 antagonistic effect of LRA with ART could be envisaged and as far as we know this
15 potential pharmacological interaction has not been assessed. In this article we have
16 analyzed the effect of 4 β -dPE A on anti-HIV activity when combined with different
17 families of types of antiretroviral drugs. Our results show a consistent synergism of LRA
18 with nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside
19 reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and even with the
20 CCR5 antagonist maraviroc. This synergistic effect would be expected for NRTIs,
21 NNRTIs and PIs, since 4 β -dPE A down-regulates CD4, CXCR4 and CCR5 receptors,
22 inhibiting HIV-1 entry and, thus, diminishing the rate of infection of T cells. However, the
23 down-regulation of CCR5 that could theoretically interfere with the maraviroc activity,

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4 1 was not found in our experiments ruling out a potential antagonism between 4 β -dPE A
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6 2 and CCR5 antagonists.”
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9 3 In summary, we show here that a 4-deoxyphorbol derivative, 4 β -dPE A, inhibits HIV
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11 4 infection through CD4, CXCR4 and CCR5 down regulation and reactivates HIV in
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14 5 resting PBMCs through PKC θ /MEK and NF- κ B activation. This last effect is independent
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16 6 of receptors downregulation, since inhibition of PKCs/MEK does not modify receptor
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18 7 expression inhibition induced by 4 β -dPE A, and, thus, other cellular targets should be
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21 8 involved. Moreover, 4 β -dPE A shows a strong synergism with HDAC inhibitor vorinostat
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24 9 as LRA and with NRTIs, NNRTIs and PIs in the antiretroviral therapy. Therefore, 4 β -dPE
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26 10 A is a new deoxyphorbol ester derivative with powerful activity as anti-latency agent and
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29 11 HIV infection inhibitor which could be a drug candidate to eradicate HIV reservoirs.
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6 2 **Conflict of interest**

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9 3 There are no conflicts of interest

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11 4 **Figure legends**

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14 5 **Figure 1. Anti-HIV-1 activity and toxicity of 4 β -dPE A.** MT-2 cells or IL-2 activated PBMCs
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16 6 were pretreated with different concentrations of 4 β -dPE A and infected with an X4 or R5 tropic
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18 7 HIV. Renilla activity (RLUs) in cell lysates was measured 48 h later. Results are expressed as %
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20 8 RLUs versus infection in the presence of the same concentration of DMSO used to dissolve the
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23 9 drug (100%). Cell Viability was assessed with CellTiter Glo luminescence viability assay.
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25 10 Citotoxicity of 4 β -dPE A and other LRAs: MT-2 cells or IL-2 activated PBMCs were pretreated
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27 11 with different concentrations of 4 β -dPE A or LRAs (vorinostat, romidepsin, panobinostat and
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29 12 prostratin). Cell Viability was assessed with CellTiter Glo luminescence viability assay 48 h later.
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32 13 Results are expressed as %RLUs versus untreated cells (100%). Long term toxicity of 4 β -dPE
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34 14 A was evaluated in pre-activated PBMCs treated with different concentrations of compound, and
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36 15 left in culture for two weeks. Every 48–72 h, cell culture was washed and treatment was added
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38 16 again. Cell viability was measured at days 2, 6, 9 and 14 by CellTiter Glo luminescence viability
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40 17 assay, and results are shown as percentage of viable cells compared to a non-treated culture
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42 18 (100%). Table shows the IC₅₀, CC₅₀ and specificity index (SI) calculated at 48 hours of
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44 19 treatment. IC₅₀: Inhibitory concentration 50% in infection (HIV) assays. CC₅₀: Cytotoxic
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46 20 concentration 50% in viability assays. CI95%: confidence interval 95%. R²: R square. All the
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48 21 result are the mean of at least 3 different replicates.
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53 22 **Figure 2. Effect of 4 β -dPE A on CD4, CXCR4 and CCR5 receptors expression.** Upper
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55 23 panels: Kinetics of expression of CD4 and CXCR4 receptors induced by 4 β -dPE A in MT-2 cells.
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57 24 Lower panels: Kinetics of expression of CD4, CXCR4 and CCR5 receptors induced by 4 β -dPE A
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59 25 in IL-2 activated PBMCs. Cells were treated with either 4 β -dPE A at 1, 10 or 100 nM or PMA
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1 (100 nM) and expression of membrane receptors was measured using specific monoclonal
2 antibodies at different points along drug treatment (2, 24 and 48 h). Results are expressed as
3 percentage of expression measured as mean intensity fluorescence (MIF) compared with
4 untreated cells (100%). All the result are the mean of at least 3 different replicates.

5 **Figure 3. HIV-1 transcriptional reactivation activity of 4 β -dPE A.** MT-2 cells and resting
6 PBMCs were transfected with pNL4.3-Luc (HIV-1). MT-2 cells, TZMbl and resting PBMCs were
7 then treated with either 4 β -dPE A (1, 10 or 100 nM) or PMA (100 nM), and cultured at 37°C for
8 48h. 100% represents the background level of expression without treatment. ANOVA analysis
9 was performed to determine the value of p (* p <0.05, ** p <0.01 *** p <0.001). All the result are
10 the mean of at least 3 different replicates.

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

20 **Figure 5. Kinetics of expression of CD4, CXCR4 and CCR5 receptors** induced by 4 β -dPE A
21 in IL-2 activated PBMCs in the presence of: A) Pan PKC inhibitor bisindolylmaleimide (Bis) B)
22 MEK inhibitor (IMEK) C) Specific PKC theta inhibitor rottlerin (Rot). Cells were treated with either
23 4 β -dPE A at 10 or 100 nM and membrane expression of receptors was measured using specific
24 monoclonal antibodies at different points along drug treatment (2, 24 and 48 h). Results are
25 expressed as percentage of expression measured as mean intensity fluorescence (MIF)

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1 compared with 4β-dPE A untreated cells without PKC or MEK inhibitors (100%). All the result
2 are the mean of at least 3 different replicates.

3 **Figure 6.** Immunofluorescence PKCθ assay. Jurkat E61 cells and PBMCs were treated with
4 PMA (100 nM) or 4β-dPE A (100 nM) or left untreated. Then, cells were permeabilized with the
5 cell fixative CytoskelFix for 10 min and incubated with phospho-PKCθ (Thr538) and Alexa Fluor
6 546 (red) goat anti-rabbit IgG antibodies and Dapi was used for nuclear staining. Images were
7 obtained with Leica DMI 4000B Inverted Microscope. The results shown are a representative
8 one of at least 3 different replicates.

9 **Figure 7. Effect of 4β-dPE A on HIV-1 LTR and NF-κB and NFAT transcription factors.**
10 Resting PBMCs were transfected with plasmids encoding a luciferase reporter gene under the
11 control of the HIV LTR or NF-κB or NFAT transcription factors. Afterwards cells were treated
12 with either 4β-dPE A at 1, 10 or 100 nM or PMA at 100 nM, and cultured at 37°C during 48h.
13 100% represents the background level of expression without treatment. T-test was performed to
14 determine the value of p (** p <0.01, *** p <0.001). All the result are the mean of at least 3
15 different replicates.

16 **Figure 8. Viral reactivation combination experiments.** A. Resting PBMCs were transfected
17 with a recombinant HIV-1 (NL4.3-Luc) and treated with serial dilutions of the drugs or their
18 combinations. EC₅₀: Effective concentration 50% in reactivation assays. CI95%: Confidence
19 interval 95%. Effective concentration 50% to effective concentration 50% combination ratio
20 (EC₅₀/EC₅₀) was used in these experiments. Combination index values at EC₅₀ were obtained
21 using CalcuSyn software. Combination index > 1.30 = antagonism, 1.10–1.30 = weak
22 antagonism, 0.90–1.10 = additive, 0.70–0.90 = weak synergy, less than 0.70 = strong synergy.
23 Values are shown as % of RLUs compared to a non-treated control (100%). B. Maximum
24 efficacy (Emax) comparisons (left panels) were performed with the top effects obtained for each

1 combination. C. Toxicity of all the combinations were tested in parallel. 100% is the value of the
2 untreated control. T test ** $p < 0.01$. All the result are the mean of at least 3 different replicates.

3 **Figure 9. Viral reactivation combination experiments.** Resting PBMCs were transfected with
4 a recombinant HIV-1 (NL4.3-Luc) and treated with a single concentration of PMA or 4β -dPE A
5 (100 nM) to be tested or a combination with an antiretroviral abacavir (ABC 1 μ M), efavirenz
6 (EFV 0.01 μ M), ritonavir (Rito 0.1 μ M), lamivudine (3TC 1 μ M), emtricitabine (FTC 1 μ M) and
7 tenofovir (TFV 1 μ M). Values are shown as % of RLUs compared to a non-treated control
8 (100%). Toxicity of all the compounds and combinations were tested in parallel (Data not
9 shown). Statistical analysis were performed using the Graph Pad Prism software (T test). All the
10 result are the mean of at least 3 different replicates.

11 **Figure 10. Antiviral combination experiments.** MT-2 cells or PBMCs were treated with
12 different concentrations of the antiretrovirals lamivudine (3TC), emtricitabine (FTC), abacavir,
13 tenofovir, efavirenz, ritonavir and maraviroc (MVC) and infected with HIV-1. After 48 hours, cell
14 culture was lysed and RLUs obtained in a luminometre. IC_{50A}/IC_{50B} ratio was used in all the
15 combinations tested. Combination index values were obtained using CalcuSyn software at EC_{50} ,
16 EC_{75} and EC_{90} (effective concentrations). Combination index > 1.30 = antagonism, $1.10-1.30$ =
17 weak antagonism, $0.90-1.10$ = additive, $0.70-0.90$ = weak synergy, less than 0.70 = strong
18 synergy. Values are shown as % of RLUs compared to a non-treated control (100%). Toxicity of
19 all the compounds and combinations were tested in parallel. All the result are the mean of at
20 least 3 different replicates.

21 **Figure 11. Transformation assay.** NIH 3T3 fibroblasts were treated with prostratin, 4β -dPE A
22 or PMA. All compounds were used at 0.1μ M concentration. As positive control, K-Ras V12
23 (p CEFL-KZ-HAK-Ras V12) was transfected by the calcium phosphate precipitation technique.
24 Morphologically transformed foci were detected after 2–3 weeks in culture by giemsa staining of

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4 1 cell cultures. As negative control, vector pCEFL-KZ-HA was transfected under similar conditions.

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6 2 The results shown are a representative one of at least 3 different replicates.

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8 3 **Figure 12. Chemical structures of 4 β -dPE A, phorbol myristate acetate (PMA) and**
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10 4 **prostratin.** The hydroxyl group in position 4 of phorbol derivatives is not needed for its activity
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13 5 as a PKC agonist and a HIV-1 reactivating agent.
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4 **4-deoxyphorbol inhibits HIV-1 infection and**
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8 **reactivates viral reservoirs through PKC/MEK**
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4 **1 ABSTRACT**
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8 2 Latent HIV reservoirs are the main obstacle to eradicate HIV infection. One strategy
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10 3 proposed to eliminate these viral reservoirs is the pharmacological reactivation of
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12 4 latently infected T cells. We show here that a 4-deoxyphorbol ester derivative isolated
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14 5 from *Euphorbia amygdaloides* ssp. *semiperfoliata*, 4 β -dPE A, reactivates HIV-1 from
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16 6 latency and could potentially contribute to decrease the viral reservoir. 4 β -dPE A shows
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18 7 two effects in the HIV replication cycle, infection inhibition and HIV transactivation,
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20 8 similarly to other phorboids agonists of PKCs such PMA and prostratin and to diterpene
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22 9 esters such SJ23B. 4 β -dPE A lacks the long side chain present in PMA, suggesting a
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24 10 non-tumour promoting activity, as our results indicate. 4 β -dPE activates HIV
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26 11 transcription at nanomolar concentrations, lower than the concentration needed other
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28 12 latency reversing agents (LRAs) such as prostratin or bryostatin. PKC θ /MEK activation
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30 13 is required for the transcriptional activity, and thus, anti-latency activity of 4 β -dPE A.
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32 14 However, CD4, CXCR4 and CCR5 receptors down-regulation effect seems to be
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34 15 independent of PCK/MEK, suggesting the existence of at least two different targets for
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36 16 4 β -dPE A. Further, NF- κ b transcription factor is involved in 4 β -dPE HIV reactivation, as
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38 17 previously shown for other PKCs agonists. We also studied the effects of 4 β -dPE A in
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40 18 combination with other LRAs. When 4 β -dPE A was combined with another PKC
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42 19 agonists such as prostratin an antagonist effect was achieved, while, when combined
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44 20 with an HDAC inhibitor such as vorinostat, a strong synergistic effect was obtained.
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46 21 Interestingly, the latency reversing effect of the combination was synergistic diminishing
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48 22 the EC₅₀ value but also increasing the efficacy showed by the drugs alone. Finally,
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50 23 combination of 4 β -dPE A with antiretroviral drugs as CCR5 antagonist, NRTIs, NNRTIs
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1 and PIs, showed a consistent synergistic effect, suggesting that the combination would
2 not induce undesirable effects on antiretroviral therapy (ART). In summary, we describe
3 here the mechanism of action of a new potent deoxyphorbol derivative as a latency
4 reversing agent candidate to decrease the size of HIV reservoirs.

5 **Keywords:** HIV-1, latency reversing agents, proteinkinases C, viral reactivation, phorbol
6 esters

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1 **1. INTRODUCTION**

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

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

18 The latency-reversing agents (LRAs) or anti-latency drugs will be the "shock" of this
19 strategy. There are several kinds of LRAs including, epigenetic modifiers, TLR agonists,
20 TCR activators, PI3K/Akt pathway modulators, NF-κB agonists, PKC agonists and
21 HDAC inhibitors [8]. PKCs agonists (prostratin or bryostatin) and HDAC inhibitors
22 (vorinostat, panobinostat or romidepsin) are the most studied LRAs. Moreover, some

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

16 PKCs agonist can activate some isoforms of protein kinases C (PKCs) by binding to its
17 regulatory C1 domain, mimicking the action of the physiological ligand diacylglycerol
18 (DAG) and inducing viral reactivation. Prostratin was the first PKC agonist studied as a
19 potential anti-latency agent [18]. Bryostatin, another PKC agonist, showed lack of
20 efficacy in the reduction of the latent reservoir in clinical trials, probably due to the low
21 doses used in the assay to avoid toxicity since systemic concentrations were found
22 undetectable [10, 19]. In this sense, some diterpenes and phorboids from
23 *Euphorbiaceae* have also been described as LRAs with antiviral properties and

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1 reactivation activity. However, slight differences in their structure lead to lack of activity
2 or a PKC isoform preference, modulating their reservoir reactivating activity [20].
3 Identification and characterization of new phorboids with different PKCs binding profile,
4 identification of other potential targets of these drugs and selection of non-tumorigenic
5 phorboids with low toxicity could be helpful for the treatment of HIV-1 latency and the
6 cure of infection.

7 Finally, as in ART, reactivation of the latent reservoir could only be achieved by the
8 combination of different drugs or antibodies able to diminish doses, and thus toxicity,
9 and to achieve cell activation enough to eliminate the reservoirs [21, 22]. In this sense, it
10 is important to point out that this therapy is intended to be used in combination with
11 ART. Some previous reports have shown that antiretroviral drugs could modulate the
12 LRAs activity and, conversely, LRAs can affect the antiviral activity of ART. For example,
13 some protease inhibitors diminish the viral reactivation obtained with LRAs [23].

14 We have reported previously the antiviral activity of 4-deoxyphorbol derivative
15 compounds isolated from *Euphorbia amygdaloides* ssp. *semiperfoliata* [24]. In the
16 present study, the mechanism of action and cellular pathways involved in the latency-
17 reversal and anti-HIV-1 effects of 4 β -dPE A, have been evaluated. This compound
18 shows antiviral activity through down-regulation of receptors and is able to induce HIV
19 reactivation. This class of compounds could be used as an adjuvant therapy to eliminate
20 the latent reservoirs of HIV-1.

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22 **2. MATERIAL AND METHODS**

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1 **Reagents**

2 4β -dPE A was isolated from *Euphorbia amygdaloides* ssp. *semiperfoliata* and provided
3 by CNRS (Centre National de la Recherche Scientifique CNRS, France). The compound
4 was solubilized in DMSO to a final concentration of 10 mM and stored aliquoted at -
5 80°C. Monoclonal antibodies (mAbs) to CD4, CXCR4 and CCR5 were supplied by
6 Becton Dickinson (Mountain View, CA, USA). Interleukin-2 (IL-2) was supplied by Chiron
7 (Emeryville, CA. USA). The inhibitor rottlerin was obtained from Alexis Co. (Lausanne,
8 Switzerland) and the inhibitors Gö6850 and Gö6976 were obtained from Calbiochem
9 (EMD Biosciences, Inc. Darmstadt, Germany). Renilla and Luciferase Assay Systems
10 and CellTiter Glo viability assay were supplied by Promega (Madison, WI, USA). DMSO
11 and MEK1/2 inhibitor PD184352 were supplied by Sigma-Aldrich (St Quentin-Fallavier,
12 France). The following reagents were obtained through the NIH AIDS Reagent Program,
13 Division of AIDS, NIAID, NIH: SAHA (vorinostat), raltegravir, lamivudine, tenofovir,
14 emtricitabine, abacavir, efavirenz and ritonavir. Romidepsin and panobinostat were
15 purchased from Selleck Chemicals (Houston, TX) and prostratin were purchased from
16 Sigma-Aldrich (St Louis, MO).

17 **Cells**

18 MT-2 cells (American Type Culture Collection, Manassas, VA, USA, Ref: CRL-2560)
19 were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 2 mM L-
20 glutamine, penicillin (50 IU/ml) and streptomycin (50 mg/ml) (all Whittaker M.A. Bio-
21 Products, Walkerville, MD, USA) and split twice a week. 293T cells, obtained from the
22 American type culture collection ATCC ref: ATCC® CRL-3216™, and TZM-bl cells (cell
23 line stably transfected with an LTR-Luc), obtained through the NIH AIDS Reagent
24 Program, Division of AIDS, NIAID, NIH: TZM-bl cells (Cat#8129) from Dr. John C.

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1 Kappes, and Dr. Xiaoyun Wu, were both cultured in DMEM medium containing 10%
2 (v/v) fetal bovine serum, 2 mM L-glutamine, penicillin (50 IU/ml) and streptomycin (50
3 mg/ml) (Whittaker) and split twice a week. PBMCs (peripheral blood mononuclear cells)
4 were isolated from healthy blood donors by centrifugation through a Ficoll-Hypaque
5 gradient (Pharmacia Corporation, North Peapack, NJ) and were suspended in RPMI
6 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and
7 antibiotics (100 mg/ml streptomycin and 100 U/ml penicillin) (all Whittaker M.A. Bio-
8 Products, Walkerville, MD, USA) before culture at a concentration of 2×10^6 cells/mL. In
9 some experiments, PBMCs were activated with IL-2 (300 IU/mL) for at least 48 h. Cells
10 were cultured at 37 °C in a 5% CO₂ humidified atmosphere. Proper informed consent
11 was obtained from each subject in accordance with the Spanish legislation on blood
12 donor regulations. Confidentiality and privacy was assured.

13 **Plasmids**

14 The vector pNL4.3-luc was generated by cloning the luciferase gene in the HIV-1
15 proviral clone pNL4.3 [25]. Plasmid pNL4.3-Ren was generated cloning the renilla gene
16 in the *nef* site of pNL4.3 and pJR-Ren plasmid was generated cloning the *env* gene of
17 HIV-1 JR_{CSF} in the pNL4.3-Ren plasmid [26]. pNL4.3-Δ*env*-Luc was obtained through
18 the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3.Luc.R⁻.E⁻ from
19 Dr. Nathaniel Landau and pcDNA-VSV encoding the G protein of VSV from Dr.
20 Arenzana-Seisdedos from Pasteur Institute [27]. The 3-*enh*-κB-ConA-luc plasmid carries
21 a luciferase gene under the control of three synthetic copies of the κB consensus of the
22 immunoglobulin k-chain promoter cloned into the BamHI site located upstream from the
23 conalbumin transcription start site [28]. The LTR-Luc plasmid carries a luciferase gene

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1 under the control of HIV-1- LTR region [29]. The expression construct pNFAT-LUC
2 containing three tandem copies of the distal NFAT-binding site of the IL-2 gene promoter
3 coupled to the IL-2 minimal promoter was kindly provided by Dr. Juan Miguel Redondo
4 (National Center for Cardiovascular Research, Madrid, Spain) [30].

5 Plasmids were amplified transforming *E. coli* DH5 α cells and cultured in LB
6 supplemented with ampicillin at 37°C for 18 hours. All plasmids were purified using
7 Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) following the manufacturer
8 instructions. DNA concentration was determined in a Nanodrop (Thermoscientific,
9 Waltham, MA, USA).

10 Viruses

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

17 Anti-HIV-1 activity evaluation by recombinant virus assay

18 The initial evaluation of 4 β -dPE A was performed in MT-2 cells infected with NL4.3-Ren
19 obtained previously by transfection in 293T cells. The assay was performed in 96 well
20 microplate seeded with 100,000 cells/well in a final volume of 200 μ l. Cell cultures were
21 infected with viral supernatants (100,000 RLU/w) and after 48 hours cell pellets were
22 lysed and luciferase activity measured in a luminometre (Berthold detection systems,

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1 Pforzheim, Germany). X4 (NL4.3-Ren) or R5 (JR-Ren) infections of IL-2 activated
2 PBMCs followed the same procedure. All the experiments were controlled with cells
3 treated with the same DMSO concentration of 4 β -dPE A. HIV-1 replication inhibition was
4 evaluated by measuring the luminescence activity or RLUs, with 100% being the
5 infection of non-treated cells. IC₅₀ was calculated with a non-linear regression formula
6 using GraphPad Prism software.

7 **Toxicity evaluation**

8 Cell toxicity was evaluated by treating mock infected cells with the same concentrations
9 of 4 β -dPE A used in the antiviral assays or with higher concentrations of 4 β -dPE A and
10 other LRAs (panobinostat, romidepsin and prostratin) or its combination with LRAs or
11 antiretroviral drugs. After 48 hours in culture, CellTiter Glo reagent (Promega, Madison,
12 WI, USA) was added and cell viability measured by RLUs quantification in a
13 luminometre. Cell viability is expressed as percentage of viable cells compared to a non-
14 treated (DMSO same concentration as compound) control (100%). Long-term toxicity
15 evaluation at different times was performed with 4 β -dPE A in IL-2-activated-PBMCs for
16 14 days. CC₅₀ was calculated with a non-linear regression formula using GraphPad
17 Prism software.

18 **Receptor expression kinetics**

19 Single-, double- or three-color immunophenotyping was performed with a FACScalibur
20 flow cytometer using CD4 (clone SK3), CXCR4 (clone 12G5) and CCR5 (clone
21 2D7/CCR5) antibodies conjugated to the appropriate fluorochrome, all obtained from
22 Becton Dickinson (Heidelberg, Germany). Background staining was assessed with the

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1 appropriate isotype- and fluorochrome-matched control mAb and subtracted. Activated
2 PBMCs or MT-2 were treated with different concentrations of 4 β -dPE A for 48, 24 and 2
3 hours. Cells were then collected and CD4, CXCR4 and/or CCR5 receptors expression
4 were evaluated. The effect of PKCs inhibitors Gö6850 (bisindolylmaleimide), rottlerin
5 and MEK inhibitor PD184352 was also studied in the expression of CD4, CXCR4 and
6 CCR5 receptors in IL-2 activated PBMCs. The analysis was performed using forward-
7 versus-side scatter dot plots. Results are shown as percentage of receptor fluorescence
8 mean intensity (%MIF) obtained by FlowJo 8.0 software.

9 **Immunofluorescence Assays**

10 For immunofluorescence assays, Jurkatt E61 cells and PBMCs were incubated in a μ -
11 Slide 8 well ibiTreat (Ibidi GmbH) for 48h, in the presence or absence of PMA or 4 β -dPE
12 A (100 nM). Then, they were permeabilized with the cell fixative CytoskelFix
13 (Cytoskeleton) for 10 min at room temperature. After washing twice with 0.1%
14 glycine/PBS, cells were incubated with phospho-PKC θ (Thr538) (Cell Signaling
15 Technology, Danvers, MA) and Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes)
16 antibodies. Washes were performed with 1X PBS–2% BSA–0.05% saponine buffer. 4',6-
17 diamidino-2-phenylindole (Dapi) was used for nuclear staining. Images were obtained
18 with Leica DMI 4000B Inverted Microscope (Leica Microsystems, Wetzlar, Germany).

19 **Evaluation of viral transcription**

20 MT-2 cells or resting PBMCs (in culture for at least 24 hours) were suspended in 350 μ L
21 of RPMI without supplements and electroporated using an Easyject plus Electroporator
22 (Equibio, Middlesex, UK) at 260 (MT-2) or 320 (PBMCs) V, 1500 mF and maximum

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1 resistance with $1\mu\text{g}/10^6$ cells of a luciferase plasmid under the control of the whole
2 genome of HIV-1 (NL4.3-Luc). Afterwards MT-2, resting PBMCs or TZM-bl (non-
3 transfected) cells were seeded in 24 well microplates and treated with different
4 concentrations of 4β -dPE A or PMA ($0.1\ \mu\text{M}$), as a reference control of HIV-1
5 transactivation, and left in culture in complete RPMI at 37°C . 48 hours later, cultures
6 were lysed with luciferase buffer and RLUs measured in a luminometer. This procedure
7 was also used for the transfection of luciferase plasmids under the control of
8 transcription factors NFAT (NFAT-Luc) or NF- κ B (3-enh- κ B-ConA-luc) or the long
9 terminal repeat of HIV (LTR-Luc) in resting PBMCs to evaluate transcriptional activity.
10 The transcriptional activity of 4β -dPE A in resting PBMCs was also studied in the
11 presence of PKCs inhibitors Gö6850 (Bisindolylmaleimide), Gö6976 or rottlerin and
12 MEK inhibitor PD184352.

13 Combination experiments

14 4β -dPE A was tested as a latency reversing agent alone and in combination with other
15 latency reversing agents as prostratin and vorinostat and a single concentration of
16 antiretrovirals abacavir (ABC $1\ \mu\text{M}$), efavirenz (EFV $0.01\ \mu\text{M}$), ritonavir (Rito $0.1\ \mu\text{M}$),
17 lamivudine (3TC $1\ \mu\text{M}$), emtricitabine (FTC $1\ \mu\text{M}$) and tenofovir (TFV $1\ \mu\text{M}$). The model
18 used to evaluate the anti-latency effect was performed in primary PBMCs isolated from
19 healthy donors. Briefly, resting PBMCs were transfected with a recombinant HIV-1
20 (NL4.3-Luc) and treated with serial dilutions or single concentrations or the drugs to be
21 tested. After 48 hours, cell culture was lysed and RLUS were obtained in a luminometer.
22 A control of the vehicle used for the dilution of the drugs was used for all the
23 concentrations tested (100%). For LRAs, EC_{50} and maximum effect (Emax) were

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1 calculated for each drug separately and EC_{50}/EC_{50} ratios were determined. Afterwards,
2 combinations experiments were performed following the same procedure with the drugs
3 alone as compared to their EC_{50}/EC_{50} combination. For antiretrovirals, a T-Test analysis
4 were performed between the reactivation rates obtained with PMA or 4 β -dPE A alone
5 and the combination with the antiretroviral drug.

6 4 β -dPE A was also evaluated in combination with antiretrovirals such as the nucleoside
7 analogue reverse transcriptase inhibitors (NRTI) lamivudine, emtricitabine and abacavir,
8 the nucleotide analogue reverse transcriptase inhibitor (NtRTI) tenofovir, the non-
9 nucleoside analogue reverse transcriptase inhibitor (NNRTI) efavirenz and the protease
10 inhibitor (PI) ritonavir. Briefly, MT-2 cells were pre-treated with serial dilutions of the
11 drugs to be tested for 30 minutes and infected with a recombinant HIV-1 (NL4.3-Ren).
12 After 48 hours, cell culture was lysed and RLUS were obtained in a luminometer. A
13 control of the vehicle used for the dilution of the drugs was used for all the
14 concentrations tested (100%). IC_{50} was calculated for each drug separately and IC_{50}/IC_{50}
15 ratios were determined. Afterwards, combinations experiments were performed following
16 the same procedure with the drugs alone as compared to their IC_{50}/IC_{50} combination.

17 **Transformation assays**

18 NIH 3T3 fibroblasts were maintained in medium DMEM supplemented with 10% calf
19 serum and treated either with SJ23B, prostratin, or PMA. All compounds were used at
20 0.1 mM concentration. As positive control, K-Ras V12 (pCEFL-KZ-HAK-Ras V12) was
21 transfected by the calcium phosphate precipitation technique. As negative control,
22 vector pCEFL-KZ-HA was transfected under similar conditions. Morphologically
23 transformed foci were scored after 2–3 weeks in culture [31]. Cells were washed with

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4 1 PBS 1x, fixed with PFA 4% for 30 min, washed again with deionized water and stained
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6 2 with Giemsa staining solution. Transformed cell foci appeared intense blue colored.
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9 3 **Statistics**

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11 4 FlowJo 8.0 software was used to analyze flow cytometry data and results are shown as
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13 5 percentage of receptor fluorescence mean intensity (%MIF) as compared to a non-
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15 6 treated control (100%). GraphPad Prism software was used to calculate effective
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17 7 concentration 50%, inhibitory concentration 50%, cytotoxic concentration 50% (EC₅₀,
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19 8 IC₅₀, CC₅₀) and Emax (top) in all the experiments (Non-linear regression, dose-response
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21 9 stimulation, log[agonist] vs response for anti-latency experiments and dose response
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23 10 inhibition, log[inh] vs response for antiviral experiments) and to represent concentration-
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25 11 response curves. Confidence intervals 95% (CI95%) and R² are provided for all the
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27 12 calculated concentrations. GraphPad Prism was also used to perform ANOVA or T-test
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29 13 analysis to determine the value of p (* p <0.05, ** p <0.01 *** p <0.001). Finally, the
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31 14 Calcusyn Software was used to evaluate combination index (CI) for each combination.
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33 15 All the experiments were performed at least in triplicates in three independent
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35 16 experiments.
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46 18 **RESULTS**

49 19 **4β-dPE A inhibits *in vitro* HIV infection with low cytotoxicity**

50 20 4β-dPE A is a phorbol derivative with special chemical characteristics, as the lack of the
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52 21 oxygen at position 4 and thus, we aimed to study its potential antiviral and latency
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54 22 reversing activity. To that end, first we used MT-2 cells infected with an X4 tropic
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56 23 recombinant virus (NL4.3-Ren) in the presence of different concentrations of 4β-dPE A.
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1 A strong inhibition of viral replication was observed at nanomolar concentrations, with an
2 IC₅₀ value of 6.51 nM. Further, when IL-2 activated PBMCs were infected under the
3 same conditions with X4 or R5 tropic HIV, a stronger inhibition of viral replication was
4 obtained, with IC₅₀ values of 0.31 and 0.24 nM, respectively (Figure 1).

6 Cell death was not observed at the concentrations tested in both, the infection assay
7 and the cytotoxicity assay. Moreover, 4β-dPE A CC₅₀ was higher than 10,000 nM (10
8 μM) in PBMCs and MT-2 cells (Figure 1). While 4β-dPE A, prostratin and vorinostat
9 showed no cell toxicity in MT-2 cells and PBMCs at 10.000 nM, a similar pattern of cell
10 toxicity were found for romidepsin and panobinostat, although panobinostat only showed
11 PBMCs toxicity at concentrations close to 10.000 nM. Since toxicity is a cause of
12 concern for anti-latency drugs, we further evaluated 4β-dPE A toxicity in a long-term
13 viability assay (14 days) in IL-2 activated PBMCs at concentrations of 0.1, 1 and 10 μM.
14 Cell viability was determined at different times along treatment. As observed in figure 1,
15 4β-dPE A was not toxic to human PBMCs even at 14 days of treatment.

17 **4β-dPE A decreases the expression of CD4, CXCR4 and CCR5 receptors**

18 Viral entry involves the interaction of HIV gp120 and gp41 with CD4, CXCR4 and/or
19 CCR5 receptors. It has been described previously the down regulation of these
20 receptors by PKCs agonists such as prostratin [32] and SJ23B [20]. Therefore,
21 expression of cell surface receptors was measured by flow cytometry in MT-2 cells and
22 IL-2 activated PBMCs. Cell cultures were stimulated with PMA or 4β-dPE A and left in
23 culture for 2, 24 and 48 h (Figure 2).

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As expected, 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 2).

4 β -dPE A induces HIV transcription

The very low number of latently HIV-infected cells *in vivo* makes purification and biochemical analysis of these cells hard to achieve. Moreover, several studies show that *in vitro* assays used to quantify the reservoir from PBMCs display a large range depending on the technique used [33]. In this work, we have used a model based on MT-2 cells and primary human resting PBMCs transfected with luciferase plasmids under the control of HIV-1 whole genome (NL4.3-Luc). We have also tested transcriptional activation in non-infected TZMbl cells, since this cell line contains in its genome integrated copies of the luciferase gene under the control of the HIV-1 promoter LTR.

4 β -dPE A activates HIV transcription in all the scenarios tested, inducing high levels of HIV-1 reactivation with low concentrations (Figure 3).

PKC theta/MEK pathway is required for 4 β -dPE A HIV transcriptional activity

To assess the PKC/MEK dependence of 4 β -dPE A-mediated antagonism of HIV latency, resting PBMCs were treated with PKCs chemical inhibitors Gö6976 1 μ M (classical PKCs inhibitor), bisindolylmaleimide 1 μ M (Gö6850, classical and novel PKCs inhibitor),

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1 rottlerin 3 μ M (PKC θ theta inhibitor) or MEK inhibitor PD184352 (IMEK) 1 μ M and HIV
2 transcriptional activity of 4 β -dPE A was also evaluated (Figure 4).

3
4 Surprisingly, novel PKC inhibition did not decreased HIV transcription induced by 4 β -
5 dPE A in a statistically significant manner. However, a tendency could be detected
6 ($p=0,1531$). However, PKC theta (rottlerin) and MEK inhibition decreased transcriptional
7 reactivation induced by 4 β -dPE A and PMA, suggesting a strong PKC theta/MEK
8 dependence.

9
10 **PKC theta/MEK pathway is not involved in 4 β -dPE A activity on receptors down-**
11 **regulation**

12 To study the specific PKC isoform involved in 4 β -dPE A receptor down -regulation, we
13 treated IL-2 activated PBMCs with PKCs chemical inhibitors, bisindolylmaleimide or
14 rottlerin, and we evaluated the 4 β -dPE A effect on CD4, CXCR4 and CCR5 receptor
15 expression. Downstream PKCs signaling in T cells may involve Raf-Mek dependent
16 activation of MAPKs, and thus MEK inhibition could also play a role in the 4 β -dPE A
17 effect. Therefore, PBMCs were treated with MEK inhibitor PD184352 and the effect of
18 4 β -dPE A in the surface cell receptor expression was also measured.

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20 Surprisingly, inhibition of PKCs or MEK did not modify the expression of CD4, CXCR4 or
21 CCR5 receptors with or without 4 β -dPE A treatment, suggesting that PKC/MEK pathway
22 is not related to the down-regulation of receptors exerted by 4 β -dPE A, and thus, to the
23 anti-HIV activity. Further, the differences of receptors expression between PKC inhibitor

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1 treated cells, MEK inhibitor treated cells and cell treated with no inhibitor were not
2 significant, suggesting that this inhibitors did not affect the receptor expression by
3 themselves (Figure 5).

4
5 **4β-dPE A induces a different pattern of intracellular distribution of PKCθ**

6 Figure 6 shows the results of PKCθ translocation in response to PMA or 4β-dPE A
7 treatment. PKCθ is the putative target of PKCs agonist in T cells [34], and we have
8 performed this experiments in PBMCs and Jurkat E61 cells by immunofluorescence.

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10 Results show a different pattern of intracellular distribution of PKCθ when PMA (used as
11 pan PKC agonist control) or 4β-dPE A are used. This results suggest that PKCθ is a
12 target of 4β-dPE A.

13
14 **NF-κB transcription factor is involved in 4β-dPE A transcriptional activity**

15 To further analyze the mechanism of 4β-dPE A reactivation activity, luciferase
16 expression vectors under the control of three tandem κB consensus repeats (NF-κB
17 Luc), NFAT (NFAT Luc) or LTR region of HIV (LTR Luc) were transfected in resting
18 PBMCs, treated with 4β-dPE A at three different concentrations (1, 10 and 100 nM) or
19 with PMA (100 nM) and luciferase activity was measured after 48 h in culture.

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22 As observed in figure 7, 4β-dPE A induced the activation of the HIV LTR and NF-κB
23 transcription factor with the highest concentration of 100 nM as PMA. However, NFAT

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4 1 was not influenced by 4 β -dPE A treatment while PMA 100 nM plus Ionomycine (Io)
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6 2 strongly increased its expression.
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11 4 **4 β -dPE A displays a synergistic effect when combined with vorinostat but not**
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14 5 **with prostratin**

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16 6 Since 4 β -dPE A is a PKC agonist, we tested its combination with another PKC agonist,
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18 7 prostratin, and with a histone deacetylase inhibitor (HDACi), vorinostat. First, we studied
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20 8 the anti-latency effect of each drug separately. As it can be seen in figure 8, all the three
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22 9 compounds showed viral reactivation in resting PBMCs. Combinations were evaluated
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24 10 among all the three drugs, including 4 β -dPE A/prostratin, 4 β -dPE A/vorinostat and
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26 11 prostratin/vorinostat and the combination effect on EC₅₀ and Emax was studied.
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33 13 4 β -dPE A displayed an EC₅₀ value of around 10 nM as transcriptional reactivator, which
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35 14 is at least 70 and 480 fold lower than the EC₅₀ of vorinostat and prostratin, respectively,
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37 15 although it was less potent than PMA (data not show, EC₅₀ 0.2 nM). The combination of
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39 16 4 β -dPE A with prostratin was antagonistic showing a clear tendency toward lowering of
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41 17 Emax value. Moreover, the combination index (CI) were above 16, showing a strong
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43 18 antagonism. On the other hand, combination of 4 β -dPE A with vorinostat showed a
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45 19 strong synergism, with a CI value of 0.20 as well as the combination of prostratin with
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47 20 vorinostat with a CI of 0.21 (Figure 8).
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55 22 **Antiretroviral drugs did not modify the reactivation capacity of 4 β -dPE A**

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57 23 To test whether antiretroviral drugs could have an effect on the reactivation capacity of
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59 24 4 β -dPE A, a single concentration combination experiment was performed (figure 9).
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1 Concentrations were selected as follows: we have used the EC₉₀ obtained from
2 reactivation experiments of 4β-dPE A and PMA and the IC₉₀ obtained from infection
3 experiments of antiretroviral drugs, since we found no reactivation effect of antiretroviral
4 drugs alone.

6 There were no significant differences among the rate of reactivation of PMA or 4β-dPE A
7 alone and the rate of reactivation of the combination with an antiretroviral drug. Only
8 abacavir showed a slight tendency to increase the viral reactivation exerted by PMA and
9 4β-dPE A, but it was not significant.

11 **4β-dPE A displays a strong synergistic antiviral effect in combination with NRTIs,**
12 **NtRTIs, NNRTIs and PIs**

13 Since the LRA treatment is designed to be administered together with ART, we
14 evaluated the effect of 4β-dPE A in the antiviral effect of antiretroviral therapy.
15 Therefore, combination experiments with different types of antiretroviral drugs were
16 performed. To that end, the entry inhibitor maraviroc (MVC), the nucleoside analogue
17 reverse transcriptase inhibitors (NRTI) lamivudine, emtricitabine and abacavir, the
18 nucleotide analogue reverse transcriptase inhibitors (NtRTI) tenofovir, the non-
19 nucleoside analogue reverse transcriptase inhibitor (NNRTI) efavirenz and the protease
20 inhibitor (PI) ritonavir were combined with 4β-dPE A using IC₅₀/IC₅₀ ratios (Figure 10).

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1 We have found that the anti-HIV effect of all the antiretroviral drugs tested in the
2 presence of 4 β -dPE A showed a strong synergism with CIs below 0.70 for all the
3 concentrations tested. However, the combination 4 β -dPE A/efavirenz showed only a
4 weak synergism with a CI of 0.78 and the combination with 4 β -dPE A/maraviroc was
5 less synergic when concentrations were higher (ED90) (Figure 10).

6
7 **4 β -dPE A do not induce cell transformation**

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

15
16 We found that both 4 β -dPE A and prostratin were unable to elicit transforming foci, while
17 transfection with KrasV12 strongly induced cell transformation. Further, PMA treatment
18 induced foci formation but to a lesser extent than KrasV12 (Fig. 11).

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21 **DISCUSSION**

22 The identification of potent natural or synthetic PKC agonists lacking tumor-promoting
23 activity and cellular proliferative activities has opened new research avenues for the

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1 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 and could potentially contribute to decrease the viral reservoir size.

5 4 β -dPE A showed two effects in the HIV replication cycle. First, it inhibits HIV infection, reported previously by us [24] and secondly it induces HIV transcriptional activation. These effects have been described for other PKCs agonists such as phorbol 12-myristate 13-acetate (PMA), bryostatin [35], prostratin [32] or SJ23B [20]. However, PMA, a phorbol diester, induced activation of HIV-1, but its potent tumor promoting activity turns its therapeutic use unacceptable [36]. Prostratin, bryostatin and SJ23B are PKCs agonists, analogs of diacylglycerol (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 has been hypothesized that the antitumor activity of PKCs agonists in general could be related to a high PKC activation leading to its degradation by the proteasome, while the natural ligand DAG or phorbol esters as PMA, with long side chains, will be inactivated before PKC degradation, promoting cellular growth. Moreover, it has been shown that phorbol esters with long acyl chain(s) such as the PMA, induced a distinct intracellular translocation pattern of PKCs and that this activity rapidly decreased with a shortening of the acyl side chain [37].

21 Thus, just like prostratin, the 4 β -dPE A, lacking the long side chain, should not display this tumor promoting activity. Short and long term toxicity experiments *in vitro* support this fact. 4 β -dPE A CC₅₀ was not reached at least at 20 μ M in PBMCs and 50 μ M in MT-

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1 2 cells, displaying a high specificity as HIV inhibitors with a specificity index greater than
2 10000 for MT-2 cells and PBMCs. Other LRAs as panobinostat or romidepsin induced
3 cell toxicity at much lower concentrations. Moreover, we did not find long term toxicity
4 after a 2 weeks treatment of PBMCs (figure 1) and transformation assays confirm the
5 lack of tumor-promoting activity (figure 11).

6 Among PKCs agonists, only prostratin and bryostatin have entered clinical trials as HIV
7 anti-latency agents although results were disappointing. The reasons of this failure are
8 not clear, but prostratin is less powerful than other phorboids [20], including 4β -dPE A,
9 and thus, doses used *in vivo* should be higher leading to toxic effects more easily. Later,
10 clinical assays with bryostatin were designed with the lowest doses possible to avoid the
11 potential toxicity of PKC agonists. However, clinical assays did not show any effect on
12 the transcription of latent HIV, due probably, as the authors stated, to low plasma
13 concentrations [10].

14 In this paper, we show that 4β -dPE A is a powerful HIV reactivation agent, with *in vitro*
15 active concentrations in the low nM range in resting PBMCs. Indeed, 100 nM
16 concentration in both cell lines was enough to induce HIV transcription at higher levels
17 than PMA at the same concentration. In PBMCs resting, just 10 nM was enough to
18 reach the same level of HIV reactivation than PMA at 100nM. Moreover, 4β -dPE A was
19 also at least 20 times more powerful than prostratin as an HIV infection inhibitor [24] and
20 this anti-HIV activity is due to the down-regulation of CD4, CXCR4 and CCR5, as
21 expected for a PKC agonist. These data showed that 4β -dPE A is at least as effective as
22 SJ23B in receptor down-regulation and more effective than prostratin.

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1 On the other hand, 4 β -dPE A was also more powerful as transcriptional reactivator than
2 vorinostat and prostratin with EC₅₀ values 70 and 480 fold lower, respectively,
3 confirming the high potency showed previously in the infectious experiments and its high
4 specificity also as LRA since its specificity index is greater than 1000 (Figure 8). The
5 classical PKCs (cPKCs) and novel PKCs (nPKCs) have two tandem C1 domains in their
6 N-terminal domain, the C1a and C1b domains, which show high binding affinities for
7 DAG, phorbol esters and other PKC activators. Interestingly, phorbol esters binding sites
8 in PKC C1 domain have been described and chemical pharmacophores identified.
9 These pharmacophores included a triad of oxygenation including the 4-hydroxyl moiety
10 [38]. However, we show here that 4 β -dPE A does not need the oxygen in position 4 to
11 display a strong PKC activating effect (figure 12).

12
13 Our results shown that the viral transcriptional effect of 4 β -dPE A depends on the
14 activation of the novel PKC θ and the mitogen-activated protein kinase kinase MEK
15 (figure 4). Previous literature showed the importance of PKC θ in T lymphocytes [34] and
16 thus, the PKC θ activation was further confirmed by immunofluorescence (figure 6). The
17 effect of phorbol esters on PKC θ and its HIV anti-latency activity have been widely
18 described. On the other hand, the effect of MEK inhibition in PKC activity has been
19 previously described [39], but less is known about its involvement in latency reactivation.
20 MEK proteins are a family of intracellular dual kinases that activates ERK/MAPK in
21 response to diverse stimuli such as IL-2 and phorbol esters. The activation of this
22 pathway is strongly involved in cell proliferation and activation of T-cells. Therefore,
23 PKC θ /MEK activation is required for the transcriptional, and thus, anti-latency activity of

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1 4β-dPE A. On the other hand, receptors down-regulation, and thus, anti-HIV activity,
2 seems to be independent of PCK/MEK (figure 5). This fact suggests a dual target of 4β-
3 dPE A in T cells, PKCθ/MEK as target for transcriptional activation (anti-latency effect)
4 and a still unknown target as an HIV infection inhibitor. Different chemical moieties could
5 be involved in each target.

6 We have further explored the mechanism of transcriptional activation. 4β-dPE A induces
7 a clear transactivation of NF-κB transcription factor (figure 7). HIV LTR contains binding
8 sites for NF-κB and its activation is crucial for the initiation of transcription of latent HIV
9 DNA. These effects are similar to those exerted by prostratin and SJ23B, the viral
10 transcriptional activation through the NF-κB transcription factor [32, 20]. Therefore,
11 phorbol esters act through the activation of HIV-1 gene transcription, although 4β-dPE A
12 seems to be a more powerful activating agent of HIV transcription, since lower
13 concentrations than the other phorbol esters are needed in the three cell lines tested
14 (SJ23B EC₅₀ between 4-8 nM; prostratin EC₅₀ between 2000-3000 nM, data not shown),
15 and thus, it could be used at lower doses in clinical assays. In fact our results show that
16 4β-dPE A is also more powerful than HDACIs, since vorinostat showed an EC₅₀ value of
17 80-120 nM. Panobinostat and romidepsin were too toxic in our assays in both PBMCs
18 and cell lines, and did not allow EC₅₀ calculation (figure 1).

19 Finally, it has been previously described that anti-latency treatment could be more
20 efficacious with combinations of different drugs. However, in clinical assays only
21 antibody-LRA combinations have been tested [21, 22] unlike drug/drug combinations.
22 Combination of drugs with different targets would be, hypothetically, synergistic. This is
23 the case of the combination of PKCs agonists such as prostratin or 4β-dPE A with an

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1 HDAC inhibitor as vorinostat. In fact, our results show that these combinations are
2 strongly synergistic, while the combinations of two PKCs agonists such as 4 β -dPE A
3 and prostratin showed a strong antagonism. Therefore, the combination of LRAs with
4 different targets would result in a synergism able to diminish the doses used to obtain
5 enough activation to reduce the reservoir size. Moreover, the combination of 4 β -dPE A
6 and vorinostat was synergistic not only lowering EC₅₀s values but also increasing the
7 Emax (figure 8). Thus, the combination of these two drugs would result in the possibility
8 of lowering doses and, at the same time, increasing the efficacy as viral reservoir
9 reactivators.

10 Since latency reversing therapy is proposed to be used together with ART in infected
11 patients, the effect of LRAs is a matter of concern if a negative effect results from this
12 treatment in two different aspects: the main one is the potential toxicity due to LRA
13 treatment on its own in patients with excellent viral control and quality of life. Besides, an
14 antagonistic effect of LRA with ART could be envisaged and as far as we know this
15 potential pharmacological interaction has not been assessed. In this article we have
16 analyzed the effect of 4 β -dPE A on anti-HIV activity when combined with different
17 families of types of antiretroviral drugs. Our results show a consistent synergism of LRA
18 with nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside
19 reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and even with the
20 CCR5 antagonist maraviroc. This synergistic effect would be expected for NRTIs,
21 NNRTIs and PIs, since 4 β -dPE A down-regulates CD4, CXCR4 and CCR5 receptors,
22 inhibiting HIV-1 entry and, thus, diminishing the rate of infection of T cells. However, the
23 down-regulation of CCR5 that could theoretically interfere with the maraviroc activity,

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1 was not found in our experiments ruling out a potential antagonism between 4 β -dPE A
2 and CCR5 antagonists.”

3 In summary, we show here that a 4-deoxyphorbol derivative, 4 β -dPE A, inhibits HIV
4 infection through CD4, CXCR4 and CCR5 down regulation and reactivates HIV in
5 resting PBMCs through PKC θ /MEK and NF- κ B activation. This last effect is independent
6 of receptors downregulation, since inhibition of PKCs/MEK does not modify receptor
7 expression inhibition induced by 4 β -dPE A, and, thus, other cellular targets should be
8 involved. Moreover, 4 β -dPE A shows a strong synergism with HDAC inhibitor vorinostat
9 as LRA and with NRTIs, NNRTIs and PIs in the antiretroviral therapy. Therefore, 4 β -dPE
10 A is a new deoxyphorbol ester derivative with powerful activity as anti-latency agent and
11 HIV infection inhibitor which could be a drug candidate to eradicate HIV reservoirs.

12

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2 **Conflict of interest**

3 There are no conflicts of interest

4 **Figure legends**

5 **Figure 1. Anti-HIV-1 activity and toxicity of 4β-dPE A.** MT-2 cells or IL-2 activated PBMCs

6 were pretreated with different concentrations of 4β-dPE A and infected with an X4 or R5 tropic

7 HIV. Renilla activity (RLUs) in cell lysates was measured 48 h later. Results are expressed as %

8 RLUs versus infection in the presence of the same concentration of DMSO used to dissolve the

9 drug (100%). Cell Viability was assessed with CellTiter Glo luminescence viability assay.

10 Citotoxicity of 4β-dPE A and other LRAs: MT-2 cells or IL-2 activated PBMCs were pretreated

11 with different concentrations of 4β-dPE A or LRAs (vorinostat, romidepsin, panobinostat and

12 prostratin). Cell Viability was assessed with CellTiter Glo luminescence viability assay 48 h later.

13 Results are expressed as %RLUs versus untreated cells (100%). Long term toxicity of 4β-dPE

14 A was evaluated in pre-activated PBMCs treated with different concentrations of compound, and

15 left in culture for two weeks. Every 48–72 h, cell culture was washed and treatment was added

16 again. Cell viability was measured at days 2, 6, 9 and 14 by CellTiter Glo luminescence viability

17 assay, and results are shown as percentage of viable cells compared to a non-treated culture

18 (100%). Table shows the IC₅₀, CC₅₀ and specificity index (SI) calculated at 48 hours of

19 treatment. IC₅₀: Inhibitory concentration 50% in infection (HIV) assays. CC₅₀: Cytotoxic

20 concentration 50% in viability assays. CI95%: confidence interval 95%. R²: R square. All the

21 result are the mean of at least 3 different replicates.

22 **Figure 2. Effect of 4β-dPE A on CD4, CXCR4 and CCR5 receptors expression.** Upper

23 panels: Kinetics of expression of CD4 and CXCR4 receptors induced by 4β-dPE A in MT-2 cells.

24 Lower panels: Kinetics of expression of CD4, CXCR4 and CCR5 receptors induced by 4β-dPE A

25 in IL-2 activated PBMCs. Cells were treated with either 4β-dPE A at 1, 10 or 100 nM or PMA

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1 (100 nM) and expression of membrane receptors was measured using specific monoclonal
2 antibodies at different points along drug treatment (2, 24 and 48 h). Results are expressed as
3 percentage of expression measured as mean intensity fluorescence (MIF) compared with
4 untreated cells (100%). All the result are the mean of at least 3 different replicates.

5 **Figure 3. HIV-1 transcriptional reactivation activity of 4β-dPE A.** MT-2 cells and resting
6 PBMCs were transfected with pNL4.3-Luc (HIV-1). MT-2 cells, TZMbl and resting PBMCs were
7 then treated with either 4β-dPE A (1, 10 or 100 nM) or PMA (100 nM), and cultured at 37°C for
8 48h. 100% represents the background level of expression without treatment. ANOVA analysis
9 was performed to determine the value of p (* p <0.05, ** p <0.01 *** p <0.001). All the result are
10 the mean of at least 3 different replicates.

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

20 **Figure 5. Kinetics of expression of CD4, CXCR4 and CCR5 receptors** induced by 4β-dPE A
21 in IL-2 activated PBMCs in the presence of: A) Pan PKC inhibitor bisindolylmaleimide (Bis) B)
22 MEK inhibitor (IMEK) C) Specific PKC theta inhibitor rottlerin (Rot). Cells were treated with either
23 4β-dPE A at 10 or 100 nM and membrane expression of receptors was measured using specific
24 monoclonal antibodies at different points along drug treatment (2, 24 and 48 h). Results are
25 expressed as percentage of expression measured as mean intensity fluorescence (MIF)

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1 compared with 4 β -dPE A untreated cells without PKC or MEK inhibitors (100%). All the result
2 are the mean of at least 3 different replicates.

3 **Figure 6.** Immunofluorescence PKC θ assay. Jurkat E61 cells and PBMCs were treated with
4 PMA (100 nM) or 4 β -dPE A (100 nM) or left untreated. Then, cells were permeabilized with the
5 cell fixative CytoskelFix for 10 min and incubated with phospho-PKC θ (Thr538) and Alexa Fluor
6 546 (red) goat anti-rabbit IgG antibodies and Dapi was used for nuclear staining. Images were
7 obtained with Leica DMI 4000B Inverted Microscope. The results shown are a representative
8 one of at least 3 different replicates.

9 **Figure 7. Effect of 4 β -dPE A on HIV-1 LTR and NF- κ B and NFAT transcription factors.**

10 Resting PBMCs were transfected with plasmids encoding a luciferase reporter gene under the
11 control of the HIV LTR or NF- κ B or NFAT transcription factors. Afterwards cells were treated
12 with either 4 β -dPE A at 1, 10 or 100 nM or PMA at 100 nM, and cultured at 37°C during 48h.
13 100% represents the background level of expression without treatment. T-test was performed to
14 determine the value of p (** p <0.01, *** p <0.001). All the result are the mean of at least 3
15 different replicates.

16 **Figure 8. Viral reactivation combination experiments.** A. Resting PBMCs were transfected
17 with a recombinant HIV-1 (NL4.3-Luc) and treated with serial dilutions of the drugs or their
18 combinations. EC₅₀: Effective concentration 50% in reactivation assays. CI95%: Confidence
19 interval 95%. Effective concentration 50% to effective concentration 50% combination ratio
20 (EC₅₀/EC₅₀) was used in these experiments. Combination index values at EC₅₀ were obtained
21 using CalcuSyn software. Combination index > 1.30 = antagonism, 1.10–1.30 = weak
22 antagonism, 0.90–1.10 = additive, 0.70–0.90 = weak synergy, less than 0.70 = strong synergy.
23 Values are shown as % of RLUs compared to a non-treated control (100%). B. Maximum
24 efficacy (Emax) comparisons (left panels) were performed with the top effects obtained for each

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1 combination. C. Toxicity of all the combinations were tested in parallel. 100% is the value of the
2 untreated control. T test ** $p < 0.01$. All the result are the mean of at least 3 different replicates.

3 **Figure 9. Viral reactivation combination experiments.** Resting PBMCs were transfected with
4 a recombinant HIV-1 (NL4.3-Luc) and treated with a single concentration of PMA or 4β -dPE A
5 (100 nM) to be tested or a combination with an antiretroviral abacavir (ABC 1 μ M), efavirenz
6 (EFV 0.01 μ M), ritonavir (Rito 0.1 μ M), lamivudine (3TC 1 μ M), emtricitabine (FTC 1 μ M) and
7 tenofovir (TFV 1 μ M). Values are shown as % of RLUs compared to a non-treated control
8 (100%). Toxicity of all the compounds and combinations were tested in parallel (Data not
9 shown). Statistical analysis were performed using the Graph Pad Prism software (T test). All the
10 result are the mean of at least 3 different replicates.

11 **Figure 10. Antiviral combination experiments.** MT-2 cells or PBMCs were treated with
12 different concentrations of the antiretrovirals lamivudine (3TC), emtricitabine (FTC), abacavir,
13 tenofovir, efavirenz, ritonavir and maraviroc (MVC) and infected with HIV-1. After 48 hours, cell
14 culture was lysed and RLUs obtained in a luminometre. IC_{50A}/IC_{50B} ratio was used in all the
15 combinations tested. Combination index values were obtained using CalcuSyn software at EC_{50} ,
16 EC_{75} and EC_{90} (effective concentrations). Combination index > 1.30 = antagonism, $1.10-1.30$ =
17 weak antagonism, $0.90-1.10$ = additive, $0.70-0.90$ = weak synergy, less than 0.70 = strong
18 synergy. Values are shown as % of RLUs compared to a non-treated control (100%). Toxicity of
19 all the compounds and combinations were tested in parallel. All the result are the mean of at
20 least 3 different replicates.

21 **Figure 11. Transformation assay.** NIH 3T3 fibroblasts were treated with prostratin, 4β -dPE A
22 or PMA. All compounds were used at 0.1 μ M concentration. As positive control, K-Ras V12
23 (p CEFL-KZ-HAK-Ras V12) was transfected by the calcium phosphate precipitation technique.
24 Morphologically transformed foci were detected after 2–3 weeks in culture by giemsa staining of

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4 1 cell cultures. As negative control, vector pCEFL-KZ-HA was transfected under similar conditions.
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6 2 The results shown are a representative one of at least 3 different replicates.

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8 3 **Figure 12. Chemical structures of 4 β -dPE A, phorbol myristate acetate (PMA) and**
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10 4 **prostratin.** The hydroxyl group in position 4 of phorbol derivatives is not needed for its activity
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12 5 as a PKC agonist and a HIV-1 reactivating agent.
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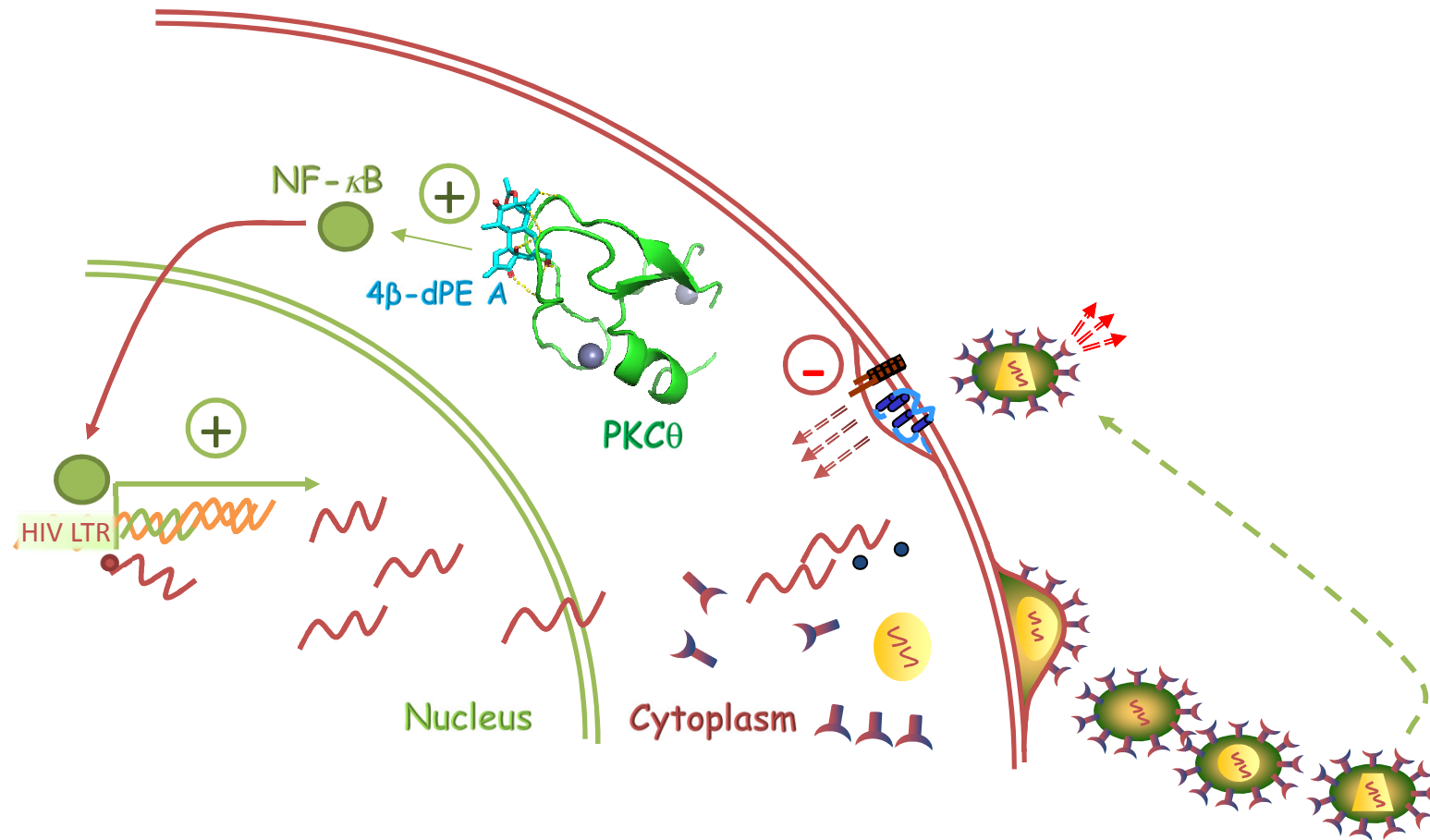
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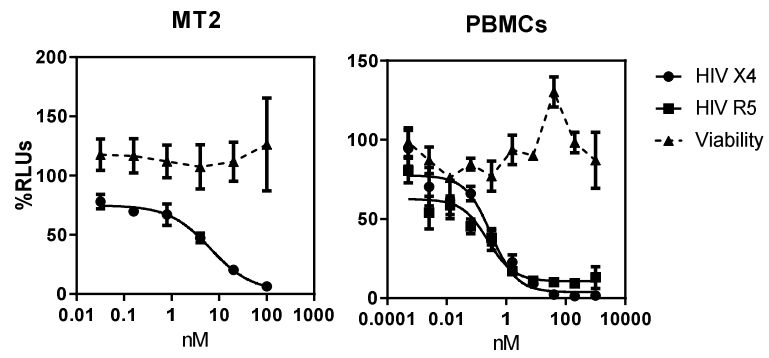
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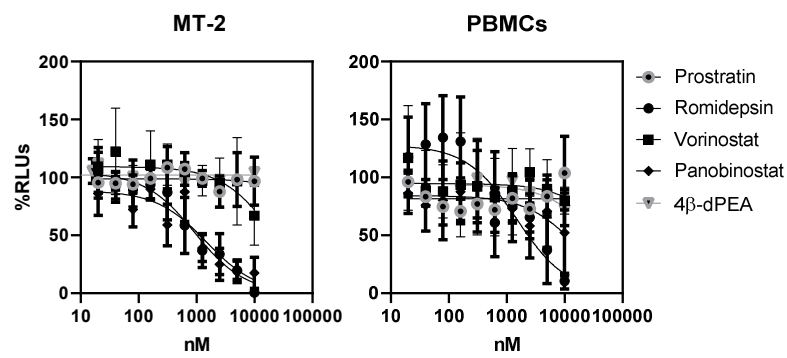
Graphical Abstract. 4-deoxyphorbol A inhibits HIV-1 infection and reactivates viral reservoirs through PKC/MEK/NF-κB activation and synergizes with vorinostat and antiretroviral drugs.

Figure



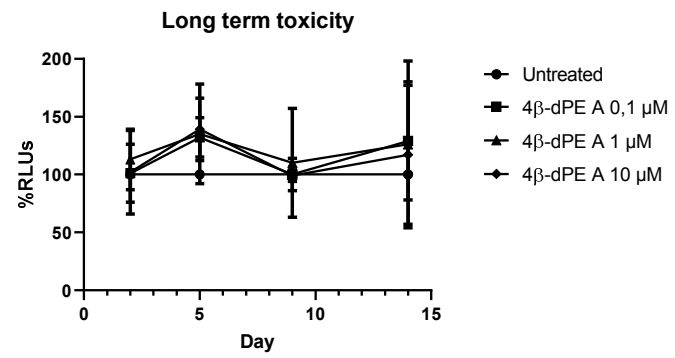
PBMCs	HIV X4	HIV R5	Viability	Specificity index SI
IC ₅₀ nM	0.31	0.24	CC ₅₀ nM	>10000 HIV X4 HIV R5
CI95%	0.16 to 0.58	0.12 to 0.50	CI95%	- >32258 >41667
R ²	0.9164	0.8950	R ²	-

MT-2	HIV X4	Viability	Specificity index SI
IC ₅₀ nM	6.51	CC ₅₀ nM	>10000 >1536
CI95%	4.18 to 10.15	CI95%	-
R ²	0.9714	R ²	-

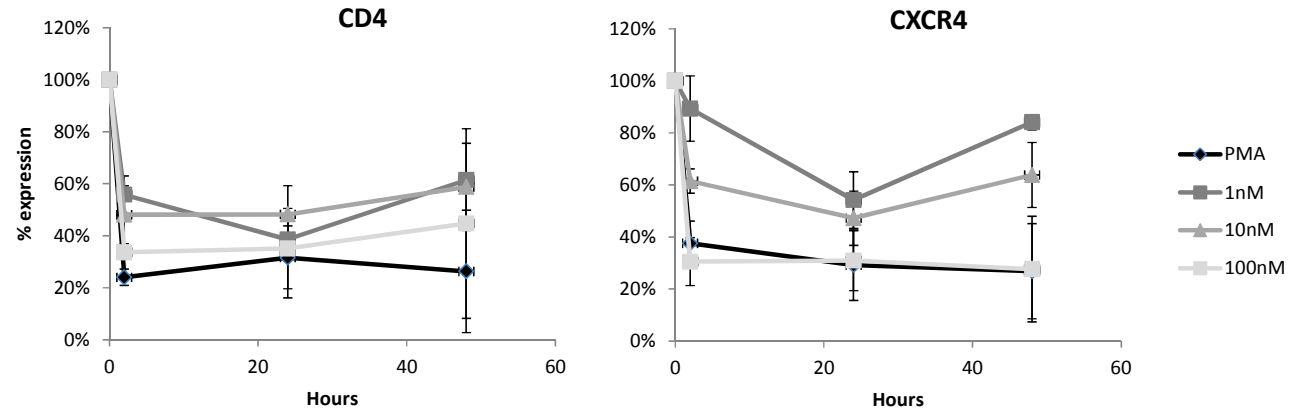


MT-2	Vorinostat	Romidepsin	Panobinostat	Prostratin	4β-dPEA
CC ₅₀ nM	>10000	932.2	1478	>10000	>10000
CI95%	-	722,5 to 1209	1039 to 2132	-	-
R ²	-	0.7988	0.681	-	-

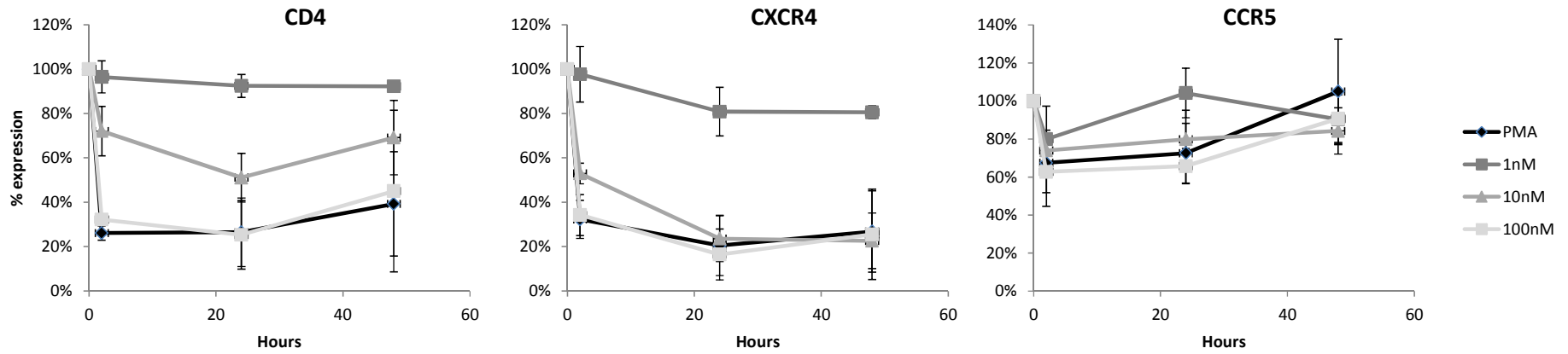
PBMCs	Vorinostat	Romidepsin	Panobinostat	Prostratin	4β-dPEA
CC ₅₀ nM	>10000	1496	≈10000	>10000	>10000
CI95%	-	929,6 to 2444	-	-	-
R ²	-	0.5955	-	-	-

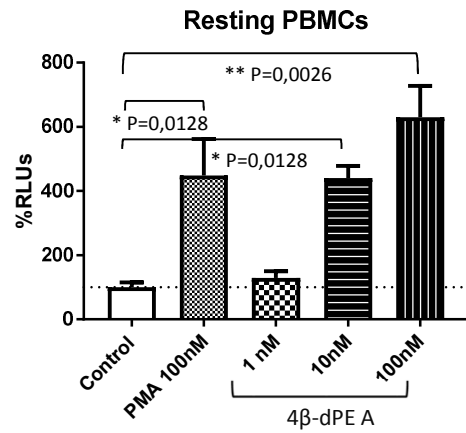
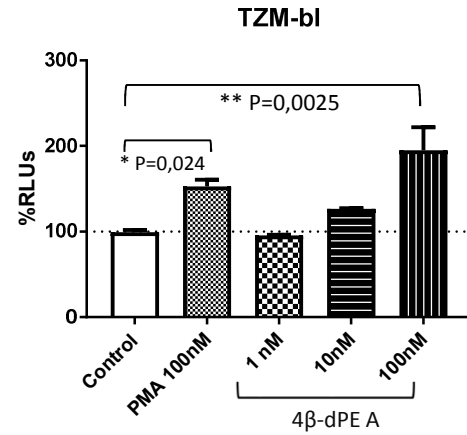
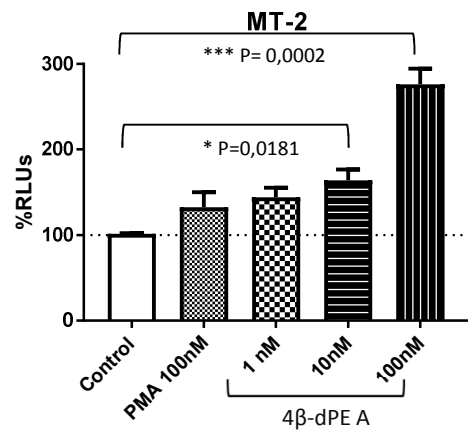


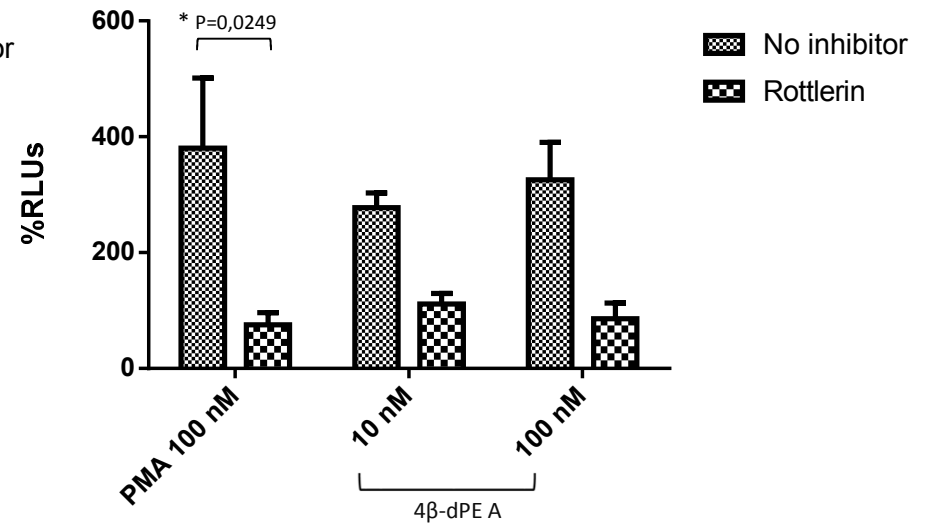
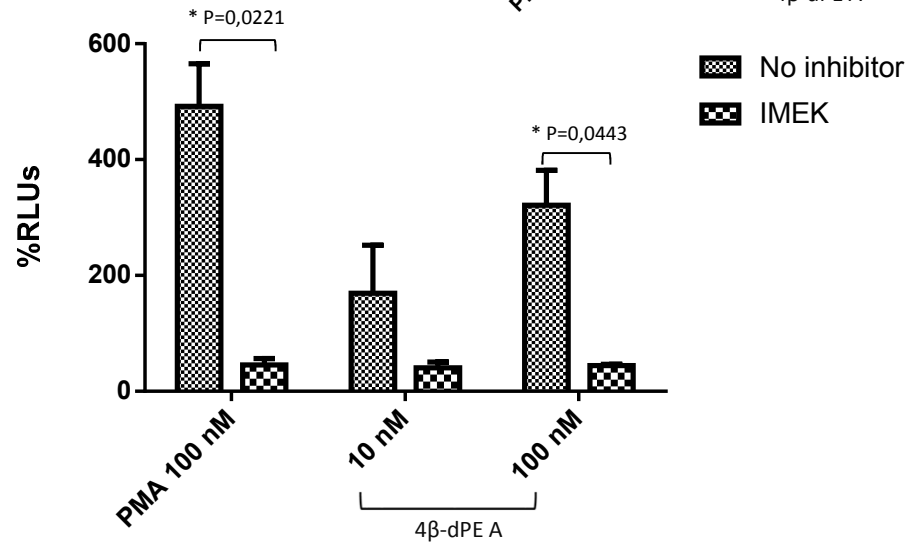
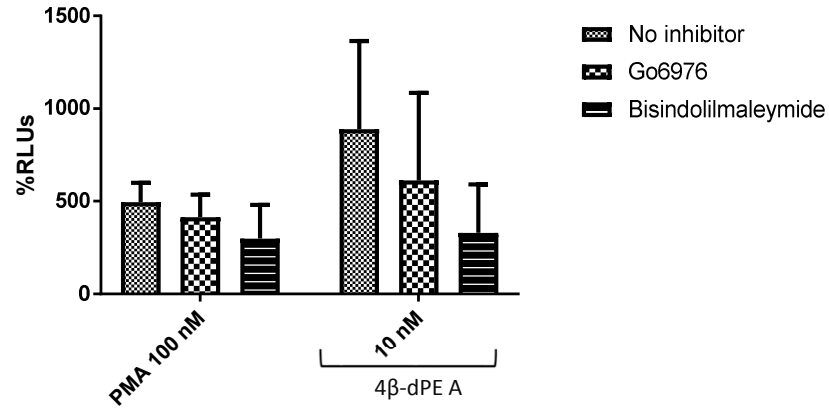
MT-2

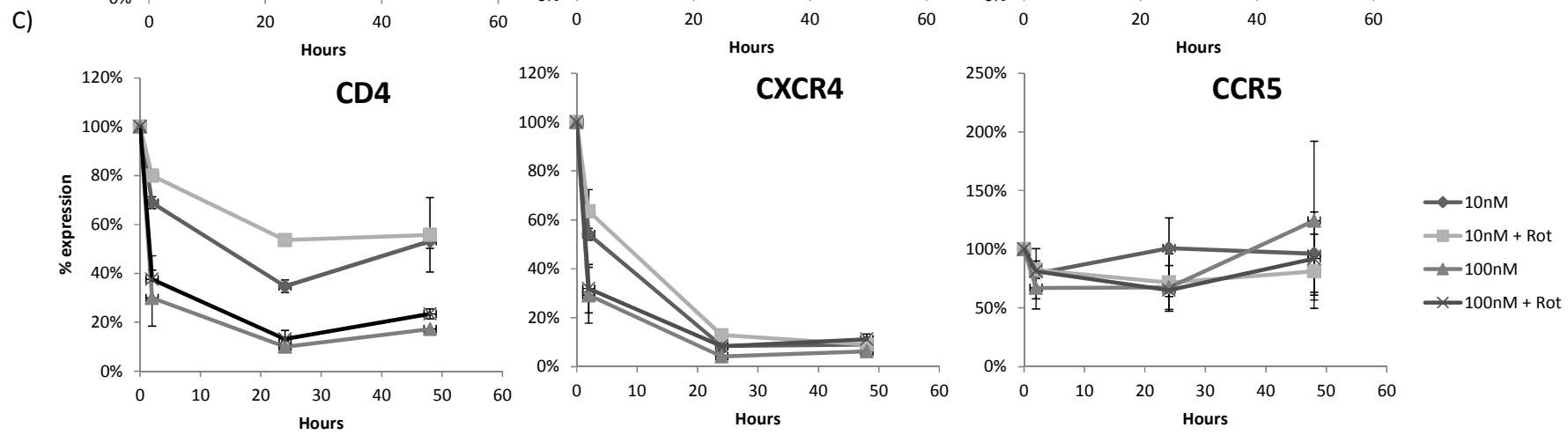
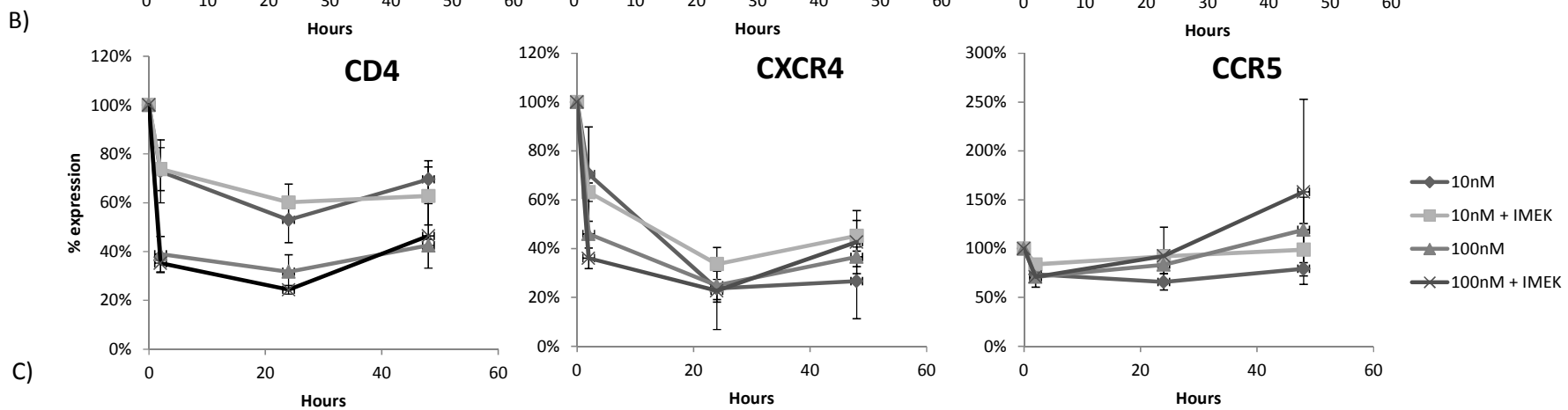
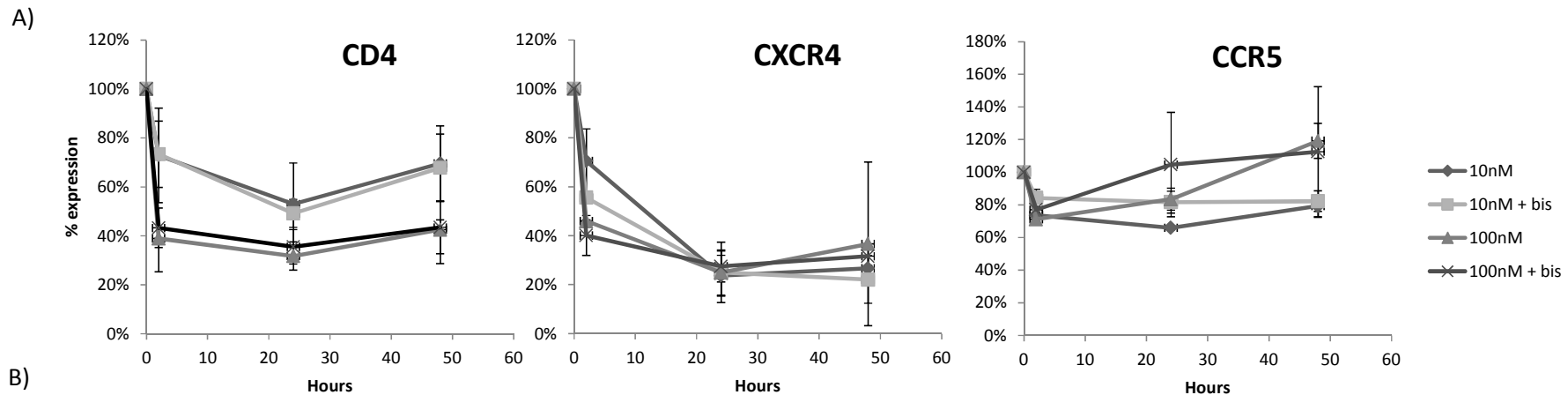


PBMCs







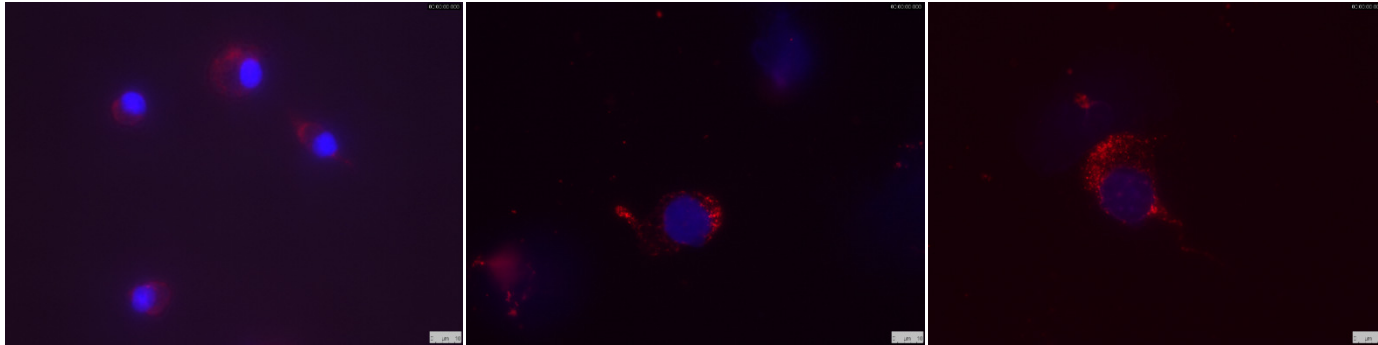


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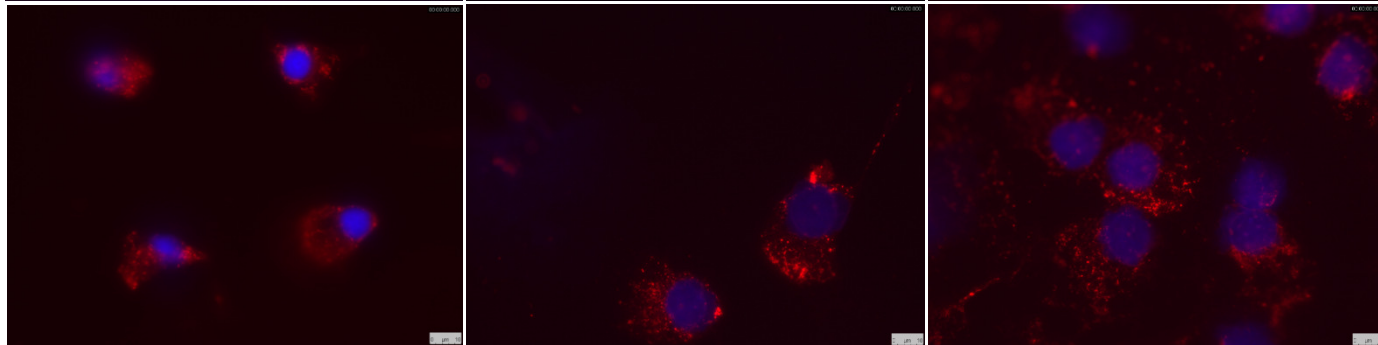
PMA 100 nM

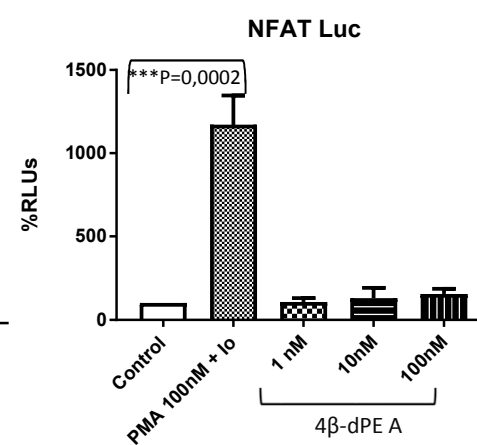
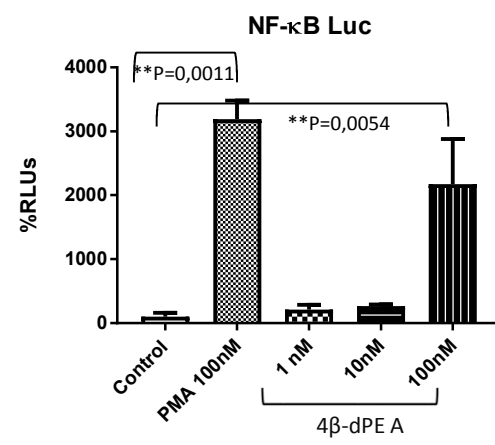
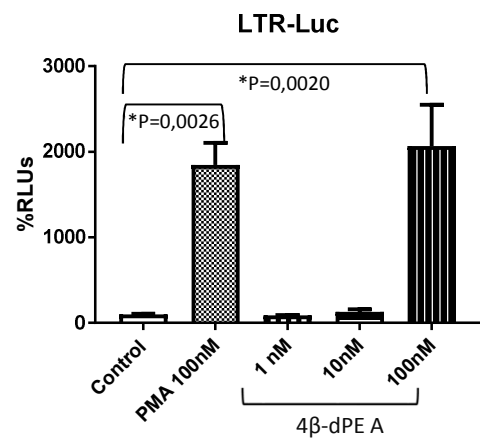
4 β -dPE A 100 nM

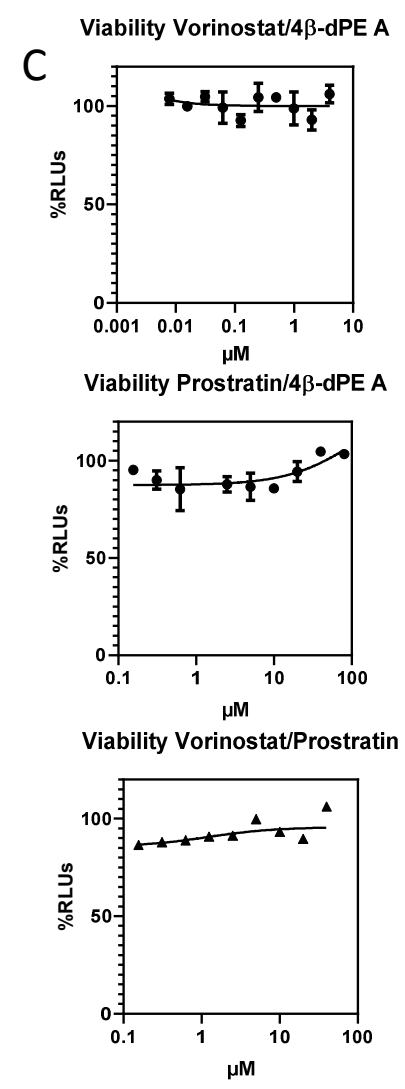
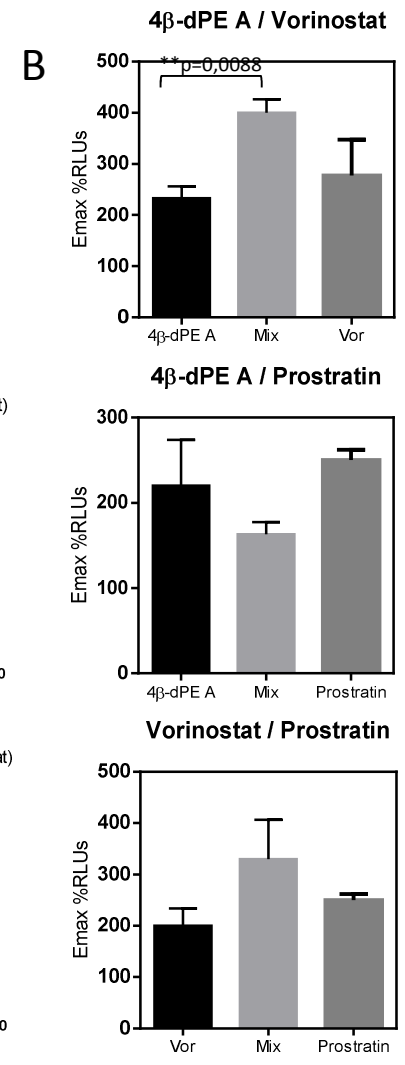
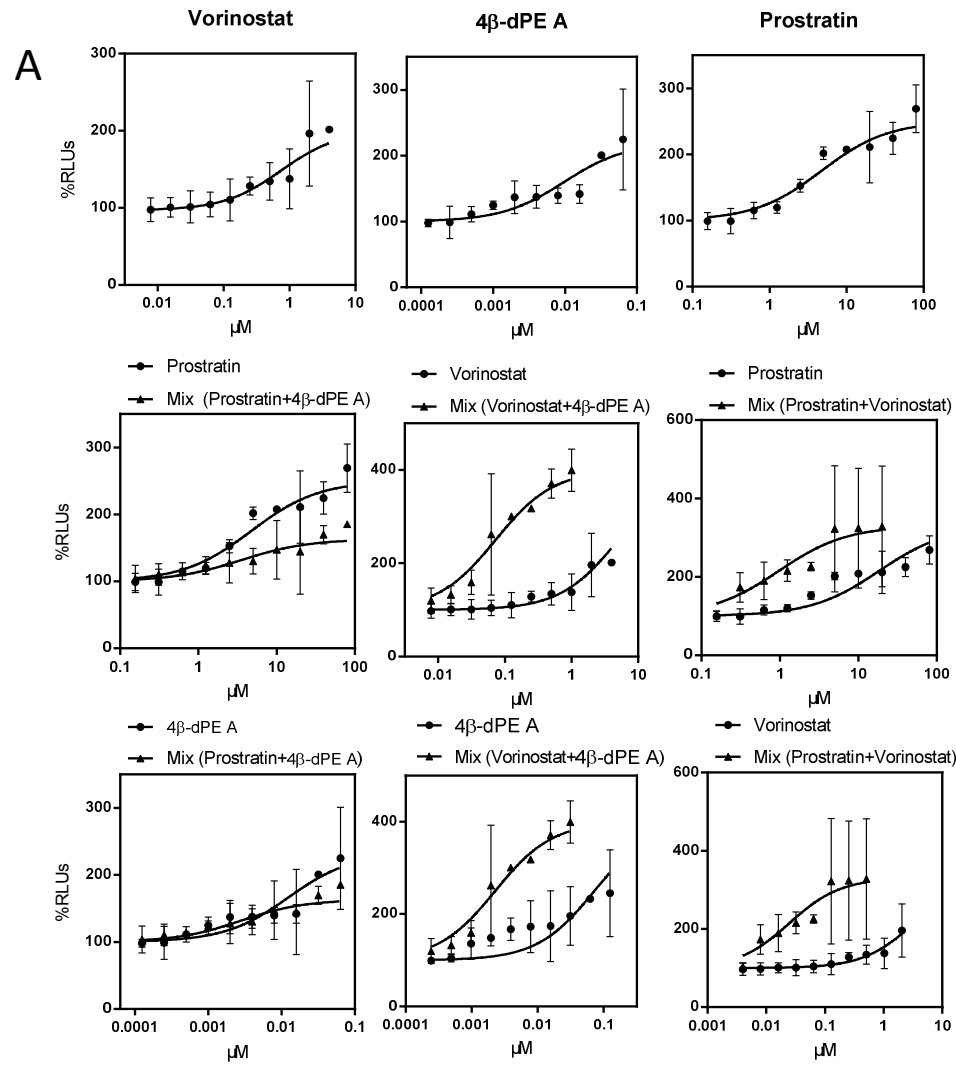
Jurkatt E61



PBMCs

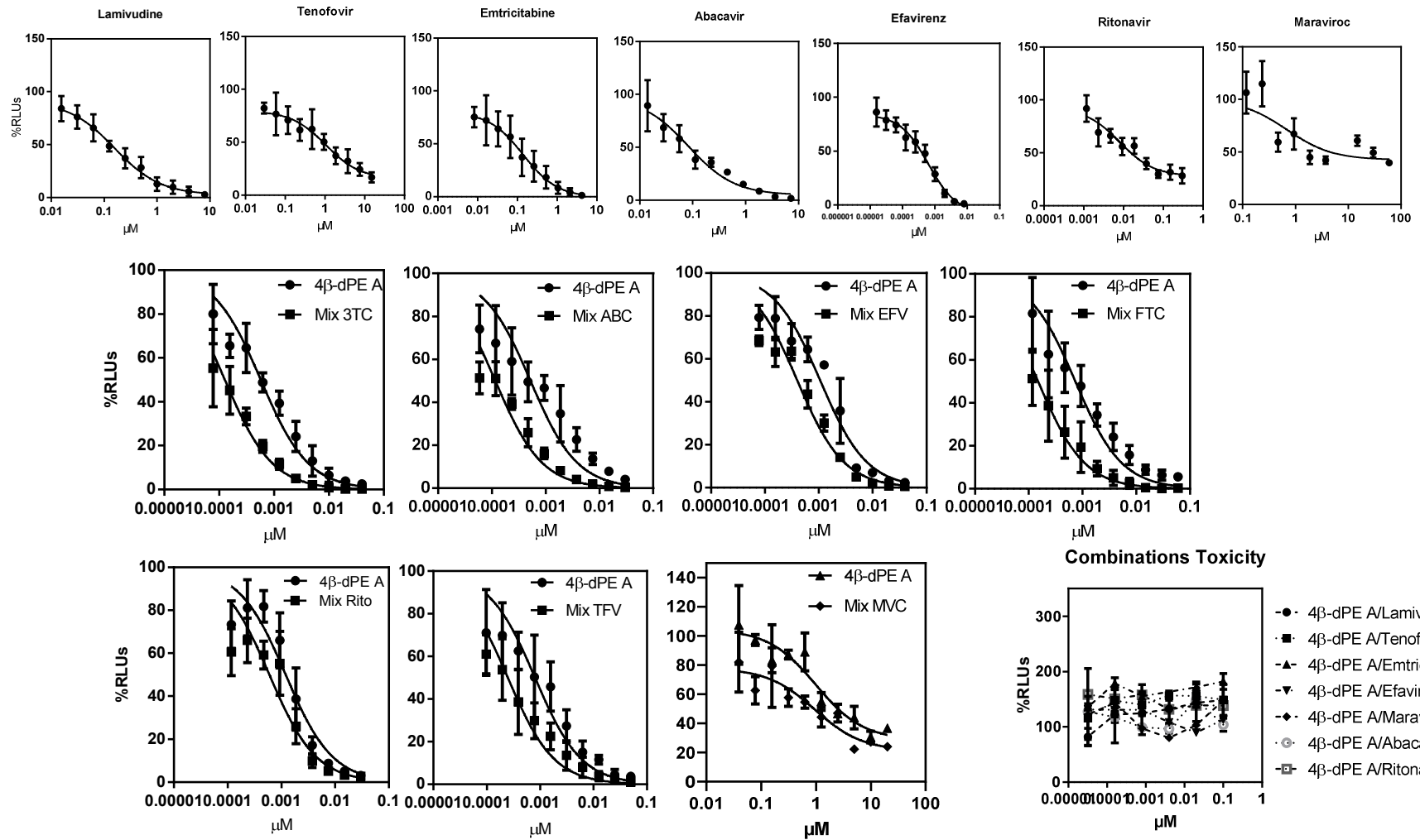






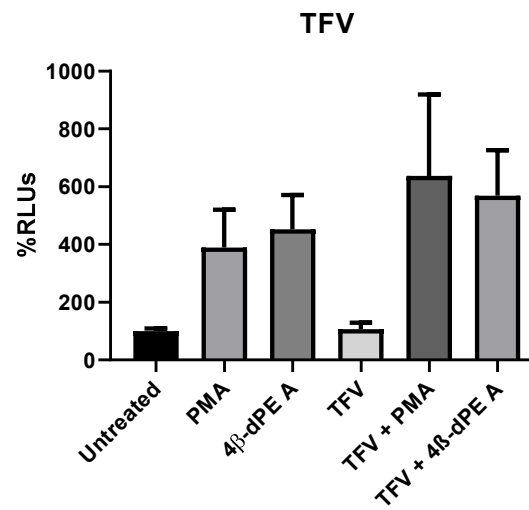
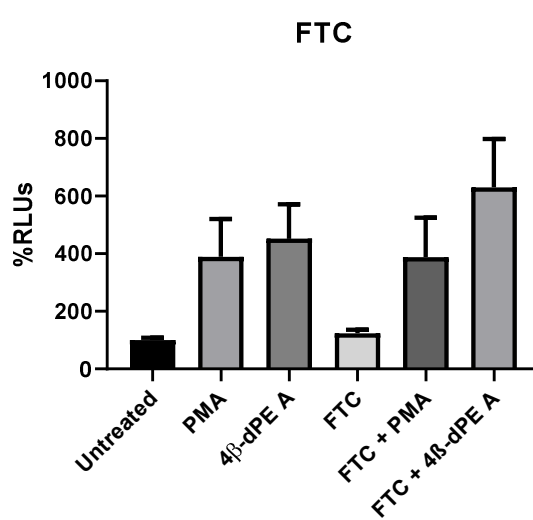
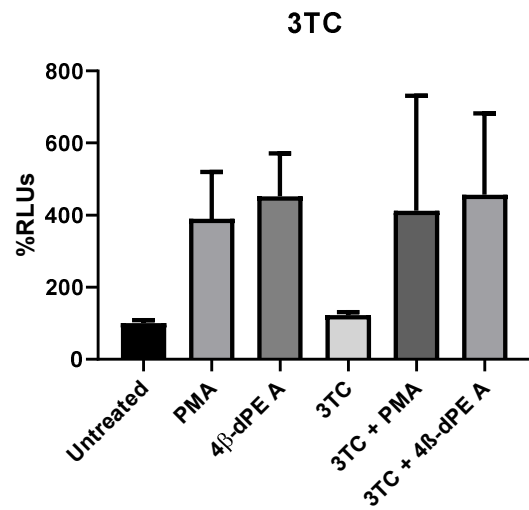
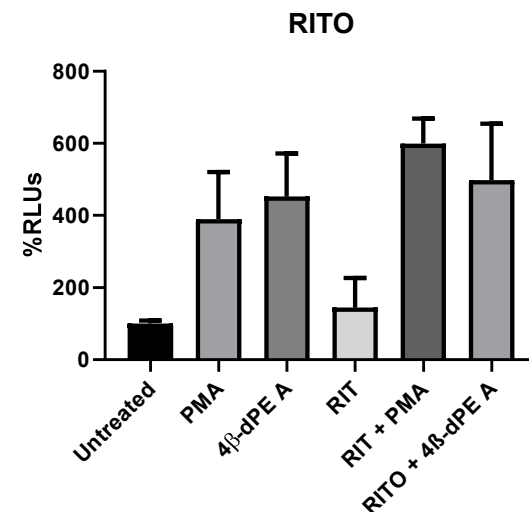
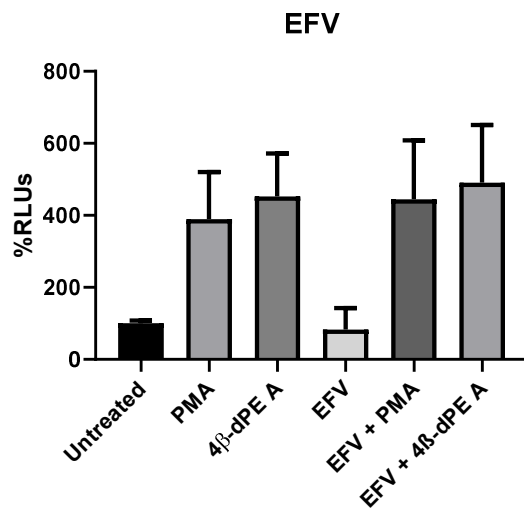
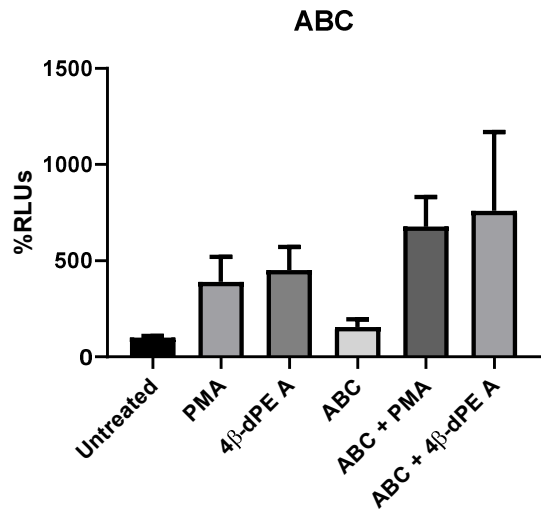
Drug combination	Combination Index Values at		
	EC ₅₀	EC ₇₅	EC ₉₀
Prostratin + Vorinostat (39.1:1)	0.20	0.23	0.25
Vorinostat + 4β-dPE A (32:1)	0.21	0.21	0.21
Prostratin + 4β-dPE A (1250:1)	16.65	110.90	739.48

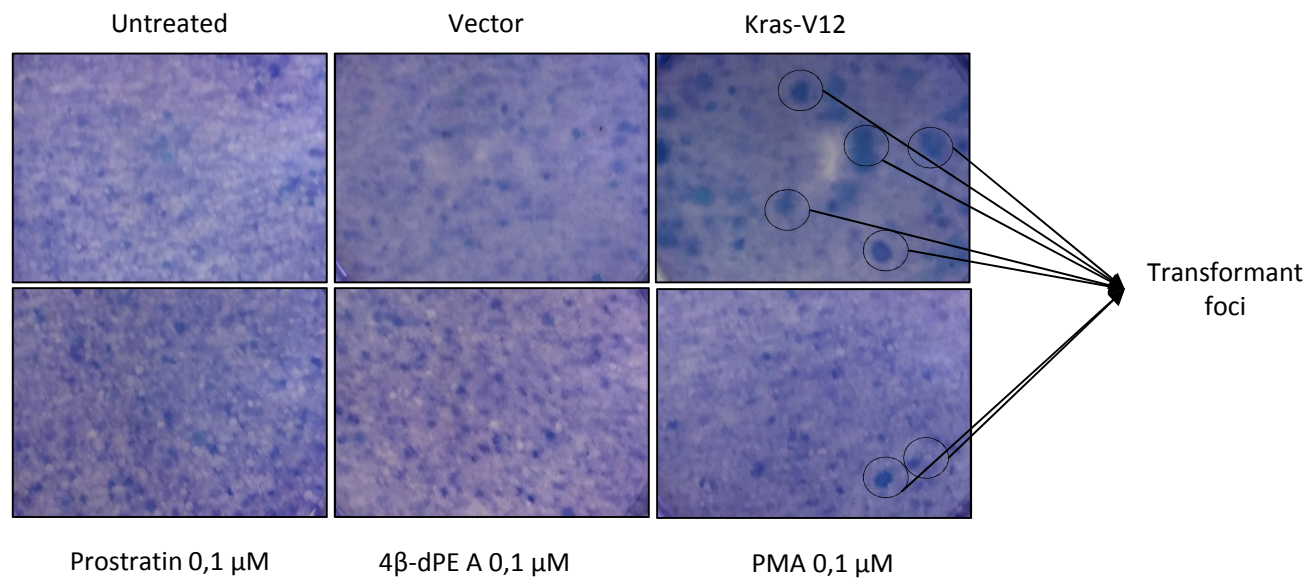
	Vorinostat	4b-dPE A	Prostratin
EC ₅₀ nM	743,5	9,7	4738,0
CI95%	433,0 to 1330,0	5,5 to 17,8	2738,0 to 8578,0
R ²	0,4922	0,609	0,8066
SI	>13	>1031	>2

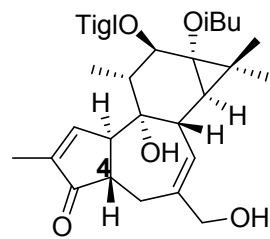


Combination Index Values at

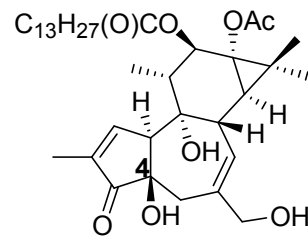
Drug combination	EC ₅₀	EC ₇₅	EC ₉₀
4β-dPE A/ABC (1:240)	0,48	0,41	0,36
4β-dPE A/3TC (1:200)	0,48	0,37	0,28
4β-dPE A/EFV (5:1)	0,78	0,84	0,90
4β-dPE A/FTC (1:70)	0,40	0,30	0,22
4β-dPE A/Rito (1:10)	0,63	0,59	0,62
4β-dPE A/TFV (1:300)	0,47	0,36	0,30
4β-dPE A/MVC (1:3)	0,58	0,69	0,87



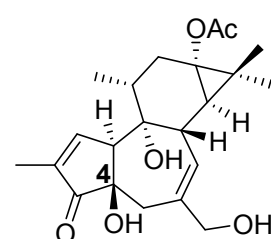




4β-dPE A



PMA



Prostratin