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2
3 **Title page**

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5 **SJ23B, A JATROPHANE DITERPENE ACTIVATES CLASSICAL PKCS AND**
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7 **DISPLAYS STRONG ACTIVITY AGAINST HIV *IN VITRO*.**
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43 **Running title:** SJ23B a new inhibitor of HIV infection
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ABSTRACT

Existence of virus reservoirs makes the eradication of HIV infection extremely difficult. Current drug therapies neither eliminate these viral reservoirs nor prevent their formation. Consequently, new strategies are needed to target these reservoirs with the aim of decreasing their size. We analysed a series of jatrophone diterpenes isolated from *Euphorbia hyberna* and we found that one of them, SJ23B, induces the internalization of the HIV-1 receptors CD4, CXCR4 and CCR5 and prevents R5 and X4 viral infection in human primary T cells at the nanomolar range. Moreover, SJ23B is a potent antagonist of HIV-1 latency. Using Jurkat-LAT-GFP cells, a model for HIV-1 latency, we found that prostratin and SJ23B activate HIV-1 gene expression, with SJ23B being at least 10-fold more potent than prostratin. SJ23B did not elicit transforming foci activity in NIH 3T3 cells but is a potent activator of PKC α and δ as measured by in vitro kinase assays and by cellular translocation experiments. By using isoform-specific PKC inhibitors we found that cPKCs are critical for SJ23B-induced HIV-1 reactivation. We also showed that both SJ23B-induced I κ B α degradation and NF- κ B activation were inhibited by the classical PKC inhibitor, Gö6976. Accordingly, SJ23B synergizes with ionomycin to translocate PKC α to the plasma membrane and to activate the NF- κ B pathway. Moreover, SJ23B activates both NF- κ B and Sp1-dependent transcriptional activities in primary T cells. We have shown that diterpene jatrophanes represent a new member of anti-AIDS agents that could be developed for mitigating HIV reactivation.

Keywords: HIV-1 and latency reactivation; SJ23B; Prostratin and phorbol esters; Receptors down-regulation; NF- κ B and PKC pathways.

1. Introduction

Highly active antiretroviral therapy (HAART) has shown to reduce viral load to undetectable levels and significantly decreased the death rate attributable to AIDS [1]. However, the persistence of HIV reservoirs, particularly in resting CD4⁺ T cells, is a major obstacle for HIV eradication [2]. Furthermore, these reservoirs are a permanent source of virus production and lead to a persistent low level ongoing replication despite HAART treatment [3]. The clearance kinetics of this latently-infected virus population remains unclear, but some conjectures suggest that eradication of latent reservoirs would require more than 70 years of suppressive therapy [4]. Moreover, patients are likely to experience drug related toxicity and may have to change therapy due to the emergence of drug resistant strains. For these reasons, the search for long-term treatment strategies for HIV-1 infection is one of the main goals of antiretroviral therapy research.

Immunoactivation therapy to reduce the latent pool of HIV by treatment with the anti-CD3 antibody OKT-3 alone or in combination with interleukin-2, substantially failed to significantly decrease the viral reservoir [5], and more recently the use of enzyme histone deacetylase inhibitors, such as valproic acid, has been proposed [6]. In addition, different small molecules including phorbol esters [7], ingenols [8] and 1,2-diacylglycerol analogs [9], can also reactivate HIV. Treatment with these molecules in combination with other antiretroviral drugs acting at different steps of the viral cycle has been suggested as a potential strategy to activate viral reservoirs and eradicate the pool of latently HIV-infected CD4⁺ T cells [10]. Non-tumor-promoting deoxyphorbol esters such as prostratin and 12-deoxyphorbol 13-phenylacetate have been directly evaluated for their ability to reactivate latent virus both in latently infected cell lines and in primary memory T cells from HIV infected patients [7, 11]. Prostratin was identified as the anti-viral constituent of the Samoan plant *Homalanthus nutans* [12] and its antiviral effect is

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3 due to two different mechanisms. On one hand, prostratin reactivates HIV-1 latency “in vitro” by
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5 protein kinase C (PKC)-dependent NF- κ B activation [12, 13], and on the other hand, down-
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7 regulates the expression of the HIV-1 receptor CD4 and the co-receptors CXCR4 and CCR5, thus
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9 avoiding the new infection of CD4⁺ cells [14].

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11 The PKC family of serine/threonine kinases plays a central role in mediating the signal
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13 transduction of extracellular stimuli, which result in the production of the second messenger 1,2-
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15 diacyl-*sn*-glycerol (DAG). The PKC family is composed of 12 members that are classified into
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17 three major subfamilies. The classical PKCs (cPKCs: α , β _I, β _{II} and γ) are Ca²⁺- and DAG-
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19 dependent, whereas the novel PKCs (nPKCs: δ , ϵ , η and θ) are Ca²⁺-independent but DAG-
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21 responsive. The atypical PKCs (aPKCs: ζ and λ /I) lack the responses to both Ca²⁺ and DAG [15].

22
23 A highly conserved cysteine-rich motif (C1 domain) in the regulatory region of the PKCs acts as
24
25 the specific receptor for the DAG signal [16]. The cPKCs and nPKCs have two tandem C1
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27 domains in their N-terminal domain, the C1a and C1b domains, which show high binding
28
29 affinities *in vitro* for DAG, phorbol esters and other PKC activators such as Ingenol and
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31 bryostatin-1 [17, 18].

32
33 The translocation of PKCs from cytoplasm to plasma membrane and other subcellular
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35 localizations is the hallmark for PKC activation, and isozyme-specific functions may result in part
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37 from a different subcellular localization of activated PKC [19]. Several studies have shown that
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39 PKC translocation is isoform-, cell type-, and activator-specific [20, 21]. Therefore, distinct
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41 patterns of localization of PKC, and therefore of access to substrates, might underlie distinct
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43 biological responses to a given PKC ligand. Clearly, the identification of potent natural or
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45 synthetic PKC agonists lacking tumor-promoter activities has opened new research avenues for
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47 the treatment of HIV-1 latency.
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3 Jatrophone diterpenes isolated from *Euphorbia hyberna* L. (Irish spurge) [22] have previously
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5 shown activity as microtubule-interacting agents [23], but there are no data about its potential
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7 anti-HIV activity. In the present study we investigate the anti-HIV-1 effects of a series of
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9 jatrophone diterpenes and we have found that SJ23B is endowed with potent antiviral activity
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11 through down-regulation of HIV receptors and induction of viral reactivation.
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16 17 **2. Material and methods**

18 19 **2.1. Reagents**

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21 The jatrophone diterpenes were isolated from *Euphorbia hyberna* L. and Terracinolide C from
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23 *Euphorbia dendroides* L. as previously described [22, 24]. mAbs to CD3, CD4, CXCR4, CD69,
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25 HLA-A,B,C and CCR5 were supplied by Becton Dickinson (Mountain View, CA, USA). IL-2
26
27 was supplied by Chiron (Emeryville, CA, USA). The anti-I κ B α mAb 10B was a gift from R. T.
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29 Hay (St. Andrews, Scotland) and the anti-tubulin mAb was purchased from Sigma Co (St. Louis,
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31 MO, USA). The inhibitor Rottlerin was obtained from Alexis Co (Lausanne, Switzerland) and the
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33 inhibitors Gö6983, Gö6976 and Gö6850 were from Calbiochem (EMD Biosciences, Inc.
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35 Darmstadt, Germany). [γ -³²P]ATP (3000 Ci/mmol) was from MP Biomedicals (Irvine, CA,
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37 USA). Prostratin was kindly provided by AIDS Research Alliance (Hollywood, CA, USA) or
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39 obtained from Alexis Co (San Diego, CA, USA). All other reagents were from Sigma-Aldrich.
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46 47 **2.2. Cells**

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49 MT-2 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI
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51 1640 medium containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, penicillin (50
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53 IU/mL) and streptomycin (50 μ g/mL) (all Whittaker M.A. Bio-Products, Walkerville, MD,
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3 USA). MT-2 cells were cultured at 37°C in a 5% CO₂ humidified atmosphere. 293 T cells were
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5 cultured in DMEM medium containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine,
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7 penicillin (50 IU/mL) and streptomycin (50 µg/mL) (Whittaker). 293T cells were cultured at 37
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9 °C in a 5% CO₂ humidified atmosphere. Jurkat-LAT-GFP is a Jurkat derived clone latently
10
11 infected with a recombinant virus containing the GFP gene driven by the HIV-LTR promoter
12
13 [25]. The cells were stimulated with the indicated compounds for 6 h and the GFP expression was
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15 analysed by flow cytometry in an EPIC XL flow cytometer (Coulter, Hialeah, FL). Ten thousand
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17 gated events were collected per sample and the fluorescence pattern was determined.
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24 **2.3. Mononuclear cell preparation**

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26 PBMCs were isolated from healthy blood donors by centrifugation through a Ficoll-Hypaque
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28 gradient (Pharmacia Corporation, North Peapack, New Jersey) and were resuspended in RPMI
29
30 1640 medium supplemented with 10% FCS (PAN Biotech GmbH, Aidenbach, Germany), 2 mM
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32 L-gutamine and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin; Whithaker, USA)
33
34 before culture at a concentration of 2×10^6 cells/ml. In some experiments, PBMCs were pre-
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36 activated with anti-CD3 antibodies (1/1000 dilution; Becton Dickinson) and IL2 (300 IU/ml;
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38 Chiron) for 48 hours.
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45 **2.4. Plasmids**

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47 The vector pNL4.3-luc was generated by cloning the luciferase gene in the HIV-1 proviral clone
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49 pNL4.3 [26]. Plasmid pNL4.3-Renilla was generated cloning the renilla gene in the *nef* site of
50
51 pNL4.3 [27] and pJR Renilla plasmid was generated cloning the *env* gene of HIV-1 JR_{CSF} in the
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53 pNL4.3-Renilla plasmid. The 3-enh-κB-ConA-luc plasmid [28] carries a luciferase gene under
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3 the control of three synthetic copies of the κ B consensus of the immunoglobulin κ -chain
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5 promoter cloned into the *Bam*HI site located upstream from the conalbumin transcription start
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7 site. The SP1-luc plasmid (a kind gift from Dr Solís-Herruzo, Hospital 12 de Octubre, Madrid,
8
9 Spain) contains two consensus sequences for SP1 cloned into the p19LUC vector. DNA for
10
11 vesicular stomatitis virus (VSV) G glycoprotein was cloned in the pcDNA3.1 plasmid (pcDNA-
12
13 VSV) [29]. The plasmids pCEFL-KZ-HA and pCEFL-KZ-HA-K-Ras V12 were previously
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15 described [30]. The pEGFP-N1 derived plasmids PKC α -GFP and PKC δ -GFP were obtained from
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17 Dr. P.M. Blumberg (NCI, MD, USA).
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24 **2.5. Anti-HIV activity evaluation**

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26 Classic antiviral assays were performed by pre treating PBMCs activated with CD3+IL2 with
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28 different concentrations of the compounds for two hours and infecting for 2 additional hours with
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30 HIV (NL4.3 strain) or recombinant HIV (NL4.3-Renilla). Afterwards, the cell culture was
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32 extensively washed with PBS, compounds were added again and left in culture for 48 hours.
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34 Supernatants were collected and HIV p24 antigen was measured using an enzyme-like
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36 immunoassay for antigen p24 detection (Innotest™ HIV Ag mAb; Innogenetics, Barcelona,
37
38 Spain). Viral production in control untreated test was at least 10 ng p24/ml (threshold of the test
39
40 was 10 pg p24/ml). The recombinant virus assay was performed as follows: Infectious
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42 supernatants were obtained from calcium phosphate transfection on 293T cells of plasmids
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44 pNL4.3-Renilla, pJR-Renilla and cotransfection of the pNL4.3-Luc-R^E (AIDS Research and
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46 Reference Reagent program, NIAID, National Institutes of Health, MD, USA), full length HIV
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48 DNA not expressing HIV envelope, and pcDNA-VSV, that express G protein of Vesicular
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50 Stomatitis Virus (VSV). These supernatants were used to infect cells, previously treated with the
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3 compounds to be evaluated for 2 hours. Anti HIV activity quantification was performed 48 or 96
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5 hours post-infection, depending on the experiment. Briefly, cells were lysed following the
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7 manufacturer instructions provided by “Renilla luciferase assay system” (Promega, Madison,
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9 WI). Relative luminescence units (RLUs) were obtained in a luminometer (Berthold Detection
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11 Systems, Pforzheim, Germany) after the addition of substrate to cells extracts. Viability was
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13 performed in cells treated in parallel and in long term cultures with similar concentrations of
14
15 compound to those used in the RV assay. Cell viability was evaluated with CellTiter Glo
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17 (Promega, Madison, WI) assay system following the Manufacturer’s specifications or by
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19 propidium iodide addition and evaluation by flow cytometry (described below). Apoptosis was
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21 evaluated with Annexin V-FITC apoptosis detection kit (BD Pharmingen, Belgium) following the
22
23 manufacturer recommendations. Inhibitory Concentrations 50 (IC₅₀) and Cytotoxic
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25 concentrations 50 (CC₅₀) were calculated using GraphPad Prism Software (sigmoidal dose-
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27 response formula).
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36 **2.6. Transfection assays**

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38 Resting PBMCs were resuspended in 350 µl of RPMI without supplements and electroporated
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40 using an Easyject plus Electroporator (Equibio, Middlesex, U.K.). PBMCs were transfected at
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42 320 V, 1500 µF, and maximum resistance with the plasmids at a concentration of 0.5 µg/10⁶
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44 cells. After transfection, cells were incubated in complete RPMI at 37° C, activated or not with
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46 different stimuli and harvested 20 hours later. Luciferase activity (RLU) was measured in a
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48 luminometer following the Manufacturer’s instructions provided by “Luciferase Assay System
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50 Kit with Reporter Lysis Buffer” (Promega, Madison, WI, USA).
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57 **2.7. CD4, CCR5, CXCR4, CD69 and HLA-1 detection by Flow cytometry**

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3 Single-, double- or three-color immunophenotyping was performed with a FACScalibur flow
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5 cytometer (Becton Dickinson, Belgium). Background staining was assessed with the appropriate
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7 isotype- and fluorochrome-matched control mAb and subtracted. Results are shown as percentage
8
9 of cells expressing the receptor or by receptor mean intensity (MI). PBMCs resting or
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11 preactivated with anti-human-CD3 (dilution 1/1000) and IL-2 (300 IU/ml) for 48 hours were
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13 analysed using forward-versus-side scatter dot plots.
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16 17 18 19 **2.8. Western blots**

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21 Jurkat-LAT-GFP cells (10^6 cells/ml) were stimulated with the indicated compounds. Cells were
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23 then washed with PBS and proteins extracted in 50 μ l of lysis buffer (20 mM Hepes pH 8.0, 10
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25 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na_3VO_4 , 5 mM NaFl, 1 mM DTT, leupeptin
26
27 1 μ g/ml, pepstatin 0.5 μ g/ml, aprotinin 0.5 μ g/ml, and 1 mM PMSF) containing 0.5% NP-40.
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29 Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA, USA) and
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31 thirty μ g of proteins were boiled in Laemmli buffer and electrophoresed in 10%
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33 SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A
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35 at 100 V; 4 °C) for 1 h. Blots were blocked in TBS solution containing 0.1 % Tween 20 and 5%
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37 non-fat dry milk overnight at 4 °C, and immunodetection of specific proteins was carried out with
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39 primary antibodies using an ECL system (GE Healthcare, Uppsala, Sweden).
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48 49 **2.9. Isolation of nuclear extracts and mobility shift assays**

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51 Jurkat-LAT-GFP cells (10^6 /ml) were stimulated with the agonists in complete medium as
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53 indicated. Cells were then washed twice with cold PBS and nuclear proteins isolated by standard
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55 procedures [31]. Protein concentration was determined by the Bradford method. For the
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3 electrophoretic mobility shift assay (EMSA), the consensus oligonucleotide probe NF- κ B, 5'-
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5 AGTTGAGG-GGACTTTCCCAGG-3' was end-labelled with [γ - 32 P]ATP. The binding reaction
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7 mixture contained 3 μ g of nuclear extract, 0.5 μ g poly(dI-dC) (GE Healthcare, Uppsala, Sweden),
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9 20 mM Hepes pH 7, 70 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 μ g/ml BSA, 4% Ficoll, and
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11 100000 cpm of end-labelled DNA fragments in a total volume of 20 μ l. After 30 min incubation at
12
13 4 $^{\circ}$ C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM
14
15 Tris-borate, 89 mM boric acid and 1 mM EDTA. Gels were pre-electrophoresed for 30 min at 225
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17 V and then for 2 h after loading the samples. These gels were dried and exposed to X-Ray film at -
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19 80 $^{\circ}$ C.
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27 **2.10. Expression of PKCs-GFP in cultured cells and visualization by confocal** 28 29 **microscopy**

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31 CHO-K1 cells were grown on 12mm round coverslips to 50-75% confluence. Transient
32
33 transfection was conducted using Jet Pei reagent (Polyplus transfection, France), following the
34
35 Manufacturer's instructions. Fluorescence became detectable 24 h after transfection, and all
36
37 experiments were performed 3 days after transfection. Prior to observation, transiently transfected
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39 CHO-K1 cells were washed twice with PBS containing 1% FCS prewarmed to 37 $^{\circ}$ C. All PKC
40
41 activators were diluted to specified concentrations in the same medium, and the final
42
43 concentration of solvent (DMSO) was always less than 1%. After 10 min of treatment the cells
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45 were fixed and analysed with a confocal microscopy (Espectral TCS-SP2-AOBS, Leica).
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53 **2.11. Transformation assays**

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3 NIH 3T3 fibroblasts were maintained in medium DMEM supplemented with 10 % calf serum
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5 and treated either with SJ23B, prostratin, or PMA. All compounds were used at 0.1 μ M
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7 concentration. As positive control, K-Ras V12 (pCEFL-KZ-HA-K-Ras V12) was transfected by
8
9 the calcium phosphate precipitation technique. Morphologically transformed foci were scored
10
11 after 2-3 weeks in culture [30]. As negative control, vector pCEFL-KZ-HA was transfected under
12
13 similar conditions.
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16 17 18 19 **2.12. PKC Kinase assay**

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21 In vitro activation of recombinant PKC α and δ (Invitrogen, Paisley, UK) was assayed by
22
23 measuring the incorporation of P^{32} from ($\gamma^{32}P$ 6000 Ci/mmol) ATP (Perkin Elmer, MA, USA)
24
25 into the specific PKC substrate peptide [QKRPSQRSKYL]. PKC activation was measured using
26
27 the PKC Assay Kit (Millipore, MA, USA) following the instructions of the manufacturer. SJ23B
28
29 or PMA were added at the indicated concentrations from the appropriated DMSO stocks, the final
30
31 concentration of the diluents not exceeding 0.2%. The assay tubes were incubated 10 min at 30 °C
32
33 and the reaction was stopped by chilling on ice. Aliquots of 25 μ l were spotted onto P81
34
35 phosphocellulose papers followed by washing three times in 0.5 % phosphoric acid and once with
36
37 acetone. The filters were transferred to scintillation vials and the bound radioactivity was read in
38
39 a scintillation counter. In every single experiment, each ligand concentration was assayed in
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41 triplicate and a dose-response curve was plotted.
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50 **3. Results**

51 **3.1. SJ23B inhibits “in vitro” infection of HIV in MT-2 cells and PBMCs**

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53 SJ23B presents a different chemical structure to those of phorbol esters and prostratin, although
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55 some structural correlations exist (Figure 1). Prostratin lacks a carbon chain at position C_{12} that
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3 makes it functionally distinct from PMA, a typical phorbol ester. This feature could explain why
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5 prostratin is not a tumour promoter, but rather protects cells from tumours induced by PMA [32].
6
7 Computer modeling analysis suggests that SJ23B lacking lipophilic chains could bind PKC, and
8
9 therefore we were interested in investigating its anti-HIV-1 activity. MT-2 cells were infected
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11 with an X4 tropic recombinant virus (NL4.3-Renilla) carrying the renilla-luciferase reporter gene
12
13 in the presence of different concentrations of compound. As observed in Figure 2A, when SJ23B
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15 was added to cultures two hours before infection, strong inhibition of viral replication was
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17 observed in MT-2 cells, at nanomolar concentrations, with IC₅₀ values of about 2 nM. When
18
19 activated PBMCs were infected under the same conditions, strong inhibition of viral replication
20
21 was also obtained, with IC₅₀ values near 20 nM. Cell death was not observed at concentrations
22
23 lower than 10 µM and CC50 was above 100 µM in both PBMCs and MT-2 cells (Figure 2A). To
24
25 further evaluate SJ23B toxicity, a long term viability assay (2 weeks) was performed, and cell
26
27 viability and apoptosis were determined at different times along treatment (Figure 2C). No
28
29 toxicity was detected during treatment, and apoptosis was only barely induced as compared to a
30
31 non-treated control of PBMCs, ruling out the potential toxicity of long term treatments in human
32
33 cells. Similar results were obtained with non preactivated resting PBMCs (Data not shown).
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35 To corroborate the anti-HIV activity, a classical p24 detection antiviral assay infecting activated
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37 PBMCs with either wild type HIV (NL4.3) or a recombinant virus (NL4.3-Renilla) was
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39 performed. As shown in Figure 2B, SJ23B is able to inhibit p24 production with similar IC₅₀
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41 values for both NL4.3 wild type virus and NL4.3-Renilla recombinant virus (IC₅₀ NL4.3: 67.42
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43 nM; IC₅₀ NL4.3-Renilla: 76.86 nM). Moreover, these IC₅₀ values are close to the IC₅₀ obtained
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45 for PBMCs in the recombinant virus assay.
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48 To compare the antiviral potency of SJ23B and prostratin, replication of X4 and R5 HIV strains
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50 was evaluated in activated PBMCs. Infections were performed with recombinant viruses (RV) in
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3 which renilla-luciferase reporter gene had been cloned. As depicted in Figure 3, inhibition of HIV
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5 infection by SJ23B is at least ten fold more potent than that of prostratin and this activity was
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7 found for infections with both, X4 and R5 tropic viruses.
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10 11 **3.2. SJ23B decreases expression of HIV receptors CD4, CCR5 and CXCR4 in PBMCs**

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13 Early stage of the HIV cycle involves the study of viral entry as one of the most suspicious steps
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15 of drug interference. To investigate this possibility, the expression of cell surface receptors
16
17 involved in HIV entry was measured. After activation of PBMCs with anti-CD3 and IL-2 for 48
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19 hours, cells were stimulated with PMA or SJ23B and left in culture for 2, 5, 24 and 48 hours.
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21 SJ23B downregulates CD4, CXCR4 and CCR5 expression in a concentration-dependent manner,
22
23 with the same pattern of down regulation as PMA. Results are shown as receptor mean intensity
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25 and percentage of expression. (Figure 4A). To rule out a non-specific effect of SJ23B on cell
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27 receptors, CD69 and HLA-1 expression in resting PBMCs was measured. PBMCs were
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29 stimulated with PMA or SJ23B and left in culture for 2, 5, 24 and 48 hours. CD69 expression was
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31 strongly enhanced by both compounds, while HLA-1 expression was not modified. Results are
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33 shown as receptor mean intensity (Figure 4B).
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43 **3.3. Antiviral effect of SJ23B is due to down-regulation of HIV receptors**

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45 To confirm that receptor expression was the only mechanism involved in HIV inhibition by
46
47 SJ23B, activated PBMCs were stimulated with PMA (0.04 μ M) or SJ23B (0.1 and 1 μ M), and
48
49 five hours later, when strong CD4, CCR5 and CXCR4 down-regulation was obtained, cells were
50
51 infected overnight with either R5, X4 HIV tropic strains or a VSV-pseudotyped viral clone
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53 lacking the HIV envelope that enters into the cell regardless of HIV receptors. Receptors
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55 expression was measured at different times during treatment (0, 4 and 7 days) and infection was
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3 measured at day 4. As shown in Figure 5B, infection of PBMCs with R5 and X4 tropic HIV
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5 strains was inhibited by SJ23B and PMA-after 4 days. Inhibition of viral replication correlated
6
7 with the capacity of SJ23B to down-regulate receptors (Figure 5A). Infection of PBMCs with
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9 VSV pseudotyped virus was not inhibited by SJ23B or PMA as expected, since these viruses
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11 enter host cells by a receptor independent mechanism. Moreover, SJ23B produces an
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13 enhancement of the infectivity of pseudotyped viruses at 0.1 and 1 μ M doses (Figure 5B),
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15 suggesting a transcriptional promoting activity similar to that previously reported for prostratin or
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17 PMA.
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24 **3.4. SJ23B activates PKC and induces HIV replication through a classical PKC pathway**

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26 The very low number of latently HIV-infected cells *in vivo* makes purification and biochemical
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28 analysis of these cells impractical. As an experimentally tractable and relevant model of
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30 postintegration HIV latency, we have employed the Jurkat-LAT-GFP clone to explore the impact
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32 of SJ23B on HIV latency. Jurkat-LAT-GFP cells contain a single, integrated lentiviral vector
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34 genome expressing GFP under the control of the HIV-1 LTR. This allows rapid assessment of
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36 HIV transcriptional activity by cytometric detection of GFP epifluorescence [25]. Thus, Jurkat-
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38 LAT-GFP cells were treated with increasing concentrations of SJ23B or prostratin and we found
39
40 that SJ23B (IC_{50} around 50 nM) is at least ten times more potent than prostratin (IC_{50} around 0.5
41
42 μ M) to induce HIV-1 reactivation from latency (Figure 6A). To further assess the PKC
43
44 dependence of SJ23B-mediated antagonism of HIV latency, we pretreated Jurkat-LAT-GFP cells
45
46 with control medium or the chemical inhibitors Gö6976 (classical PKCs inhibitor), Gö6850
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48 (classical and novel PKCs inhibitor), Gö6983 (pan-PKC inhibitor) and Rottlerin (PKC δ inhibitor)
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50 at the indicated concentrations. Gö6976, Gö6850 and Gö6983 strongly inhibited GFP expression
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52 induced by SJ23B, further implicating a PKC-dependent signalling step in this response. In
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3 contrast, the PKC δ inhibitor Rottlerin did not affect phorbol-induced GFP expression, ruling out
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5 the involvement of this PKC in HIV-1 reactivation, at least in Jurkat-LAT-GFP cells (Figure 6B).
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7 Next, we analysed the ability of SJ23B to activate kinase activity *in vitro* using a substrate
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9 peptide that can be efficiently phosphorylated both by PKC α and PKC δ . We show that SJ23B
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11 activates both PKC isoenzymes in a concentration-dependent manner although it was more potent
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13 for PKC α activation compared to PKC δ (Figure 6C).
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19 **3.5. SJ23B activates NF- κ B through a classical PKC dependent pathway**

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21 Since prostratin and other PKC agonists antagonise HIV-1 latency by activating the canonical
22
23 pathway for NF- κ B activation, we explored the signalling pathway activated by SJ23B in Jurkat-
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25 LAT-GFP cells that mediates activation of the latent HIV proviruses. The cells were stimulated
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27 with SJ23B (1 μ M) in the absence or presence of the indicated PKC inhibitors, and degradation
28
29 of the NF- κ B inhibitor I κ B α and the binding of NF- κ B to DNA were investigated by Western
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31 Blots and gel retardation assays respectively. We found that SJ23B induced the degradation of
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33 I κ B α and the binding of NF- κ B to DNA and both activities were completely inhibited by the
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35 presence of classical PKCs inhibitors (Fig. 7). As expected, Rottlerin did not affect either SJ23B-
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37 induced I κ B α degradation or NF- κ B binding to DNA, confirming that PKC δ is not involved in
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39 the HIV-1 antagonizing activity of SJ23B.
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48 The translocation of PKC from the cytoplasm to subcellular localizations is the hallmark for PKC
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50 activation [33], and their isozyme-specific functions may result in part from differences in
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52 subcellular localization [20]. The classical PKCs are Ca²⁺- and DAG-dependent and we
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54 hypothesized whether SJ23B could synergise with increased intracellular calcium levels in the
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3 cellular redistribution of PKC α . In order to explore the specificity of PKC α translocation in
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5 response to PMA and SJ23B in the absence or presence of the calcium ionophore ionomycin,
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7 CHO-K1 cells were transiently transfected with a construct containing the full length open ready
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9 frame of PKC α fused to the GFP gene. In the absence of stimuli, PKC α was scattered in the
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11 cytoplasm and after PMA treatment the protein was mainly translocated to the plasma membrane.
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13 SJ23B also induced redistribution of the PKC α to the plasma membrane that was further
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15 enhanced by the presence of ionomycin (0.5 μ M) (Figure 8A). Conversely, SJ23B alone or in
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17 combination with ionomycin did not induce PKC δ translocation to the plasma membrane (data
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19 not shown).
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26 To correlate the effects of SJ23B in the translocation of PKC α in CHO cells with the NF- κ B
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28 activity in Jurkat-LAT-GFP cells, we treated this cell line with increasing concentration of SJ23B
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30 in the absence or presence of ionomycin (0.5 μ M) and the binding of NF- κ B to DNA was
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32 analysed by electrophoretic mobility shift assays. In Figure 8B it is shown that elevated
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34 intracellular levels cooperate with SJ23B to increase the DNA binding activity of NF- κ B.
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36 Altogether our results indicate that the major pathway for SJ23B to antagonize HIV-1 latency
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38 involves the activation of PKC α , which in turn activates the canonical NF- κ B pathway.
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45 **3.6. SJ23B do not induce cell transformation**

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47 There is a general agreement that only phorbol esters inducing a sustained PKC translocation to
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49 the cell membrane are endowed with tumour promoter activity. To demonstrate that SJ23B lacks
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51 tumour promoter activity we performed transforming cell assays in NIH 3T3 fibroblasts [34], and
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53 we found that both SJ23B and prostratin were unable to elicit transforming foci, while treatment
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3 with PMA to the same concentrations, or transfection with KrasV12, strongly induced cell
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5 transformation (Figure 9).
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8 9 10 **3.7. SJ23B activates HIV-1 transcription and induces NF- κ B- and Sp1-dependent gene** 11 **expression in peripheral blood mononuclear cells**

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13 To further assess the effect of SJ23B in HIV-1 transcriptional activation in primary cells, a full-
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15 length HIV-DNA plasmid containing the luciferase gene instead of *nef* (pNL4.3-Luc) was
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17 transfected into resting PBMCs. In Figure 10 it is shown that luciferase activity reflecting HIV
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19 reactivation was induced when treated with PMA and two different doses of SJ23B. Prostratin
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21 induces luciferase activity at 1 μ M, but not at 0.1 μ M. Different regulatory sequences could also
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23 be implicated in SJ23B-induced HIV reactivation. To further analyze the transactivation
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25 mechanism, luciferase expression vectors under the control of three tandem κ B consensus repeats
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27 (3-enhancer- κ B-ConA-Luc) or Sp1 were transfected into PBMCs and luciferase activity was
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29 measured after 20 hours (Figure 10). Induction of the luciferase gene under the control of three
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31 κ B consensus repeats was observed when cells were activated with SJ23B, at higher levels than
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33 prostratin-induced stimulation. Also, SJ23B seems to induce activation of SP1 (Sp1-Luc)
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35 similarly to prostratin. Finally, transactivation of NFAT transcription factor was not detected
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37 when PBMCs were stimulated with SJ23B or prostratin (data not shown). Therefore, SJ23B
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39 seems to be a more effective promoter transactivator than prostratin in primary cells mainly
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41 through activation of NF- κ B.
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53 **3.8. Acetylation at position C-16 is required for the HIV-1 latency antagonizing activity** 54 **of jatrophone diterpenes**

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3 A series of jatrophone diterpenes were also tested on Jurkat-LAT-GFP cells for comparison with
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5 SJ23B. Jatrophone polyesters are highly functionalized, and occur as complex mixtures. They are
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7 therefore difficult to obtain in amounts sufficient to sustain a systematic structure-activity study.
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9 Nevertheless, comparison of a series of jatrophanes available by isolation from two
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11 Mediterranean spurge (*E. hyberna* and *E. dendroides*) shows that acetylation of the 15-hydroxyl
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13 and acetoxylation at C-2 are critical for the HIV-1 latency antagonising activity. Both
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15 modifications are relatively redundant for inhibition of P-glycoprotein, the only molecular target
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17 so far identified for this type of compounds [24]. This observation is non surprising since, given
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19 the wide diversity of functional groups present in this type of compounds, countless possibilities
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21 of specific interaction with a macromolecular target can exist (Figure 11).
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28 29 **4. Discussion**

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31 AIDS continues to be a global health threat, although new therapies, especially the highly active
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33 antiretroviral therapy (HAART), have reduced dramatically the death rate attributable to HIV
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35 infections. However, HAART associated problems such as resistance emergence and drug
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37 toxicity, makes the eradication of the virus essential in order to end long term treatments,
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39 currently sustained for life. This imperative objective can only be achieved by eradicating HIV
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41 reservoirs completely from infected patients.
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45 The identification of potent natural or synthetic PKC agonists lacking tumour-promoter and
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47 cellular proliferative activities has opened new research avenues for the treatment of cancer and
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49 HIV-1 latency. Bryostatin-1 has been shown to regulate protein kinase C (PKC) activity and can
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51 potentially inhibit tumour invasion, angiogenesis, cell adhesion, and limit the development of
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53 multidrug resistance [35]. Preclinical studies of bryostatin-1 have demonstrated both *in vitro* and
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55 *in vivo* anti tumour activity [36]. On the other hand, the capacity of prostratin to behave as an *in*
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3 *in vivo* agent to purge latent HIV-1 proviruses has raised considerable interest, owing to a potential
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5 clinical application in combination with HAART to eradicate HIV-1 infection [37]. However,
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7 relatively high concentrations of prostratin are required to reactivate HIV-1 latency and it has
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9 been suggested that prostratin may have negative side effects and therefore it is unlikely that
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11 high-doses or long term treatment would be well tolerated [38]. In this study we analysed a series
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13 of natural jatrophone diterpenes derivatives and explored the mechanism by which these
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15 compounds antagonized HIV-1 latency. The strong anti-HIV activity at the nanomolar range was
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17 due to downregulation of HIV-1 receptors. Furthermore, we show that SJ23B can reactivate HIV-
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19 1 from latency and could potentially contribute to a decrease in viral reservoirs. Therefore,
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21 similar to prostratin, SJ23B can qualify for the treatment of HIV-1 latency.
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26 SJ23B is a diterpene with a jatrophone skeleton and a lactone moiety that was isolated from
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28 *Euphorbia hyberna* [22], a Mediterranean plant specimen without previous established antiviral
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30 activity. Although its chemical structure is quite different from phorbol esters, some biological
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32 correlations are possible, as our data indicate that SJ23B antagonizes HIV-1 latency through
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34 activation of classical PKCs in HIV-1 latently infected Jurkat cells. We do not yet know how
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36 SJ23B interacts with the phorbol binding domain of PKC, but the lack of lipophilic chains made
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38 this compound a potent PKC activator that does not allow a sustained translocation of cPKCs and
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40 nPKCs to the plasma membrane (data not shown). Thus, SJ23B is likely to belong to the class of
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42 PKC activators lacking tumour promoter activity that was confirmed on the transforming foci
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44 assays.
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51 As previously described, prostratin enhanced SP1 and NF- κ B pathway dependent transcription
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53 through a mechanism dependent on protein kinase C (PKC) [13]. To analyze transactivator
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55 activity of SJ23B in primary cells, resting PBMCs were transfected with a full length HIV clone
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57 driving the expression of luciferase as a reporter gene. Induction of HIV transcription by SJ23B
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3 was observed even at 0.1 μM , whereas higher doses of prostratin (1 μM) were required to induce
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5 HIV expression. To further study the effect on HIV promoter, capability to induce transactivation
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7 of SJ23B, prostratin and PMA were studied with a luciferase expression vector under the control
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9 of consensus sequences for NF- κB and SP1. Results showed that SJ23B induces a clear
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11 transactivation of NF- κB (3-Enh- κB -ConA-Luc) and SP1 transcription factors, although the
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13 former was strongly induced. Altogether our results demonstrate that, similarly to prostratin,
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15 SJ23B targets the NF- κB and SP-1 transcription factors and is a potent activator of HIV-1 gene
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17 transcription in both primary cells and in HIV-1 latently infected cell lines, with SJ23B being at
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19 least 10-fold more potent than prostratin.
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24 We show here that SJ23B inhibits *in vitro* recombinant HIV (NL4.3-Renilla) infections of a
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26 lymphocyte cell line (MT-2) at the nanomolar range, leading us to test it in a more physiological
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28 system. Activated PBMCs infection experiments with recombinant viruses and classical HIV-
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30 p24-gag antigen assays, corroborate the antiviral activity of SJ23B, lacking cell toxicity, since
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32 CC_{50} was not reached even at 100 μM . Thus, the interest of this compound as a potential anti-
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34 HIV agent is based on its powerful activity, since it shows a high specificity index, greater than
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36 those obtained with similar cell lines and viral strains for AZT [39].
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41 Data from preliminary assays implies that SJ23B does not display virucidal activity (data not
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43 shown) and its target should be previous to retrotranscription, suggesting a direct interference of
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45 SJ23B on viral entry. This process is mediated by viral glycoproteins and host cell receptors
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47 CD4, CXCR4 and/or CCR5. Expression of these receptors in cell surface is variable and
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49 particularly CXCR4 expression on the surface of PBMCs is low when freshly isolated, increasing
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51 to near 90% when maintained at least 12 hours without stimuli [40]. The effect of SJ23B on cell
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53 surface receptors was studied using PMA as reference compound. Results showed that SJ23B
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55 down-regulates CD4, CXCR4 and CCR5 receptors in preactivated PBMCs. This down-regulation
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3 should be associated with an inhibition of HIV infection by even R5 or X4 tropic viruses.

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5 Therefore, an RV assay is performed infecting PBMCs with X4 or R5 tropic viruses. As

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7 expected, SJ23B inhibits HIV replication in infections with both tropic viruses. These

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9 experiments suggest that antiviral activity of SJ23B is due to internalization of receptors.

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11 In summary, the persistence of latent reservoirs of human immunodeficiency virus type 1 (HIV-

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13 1) represents a major barrier to virus eradication in patients treated with highly active

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15 antiretroviral therapy. Immunoactivation therapy to reduce the latent pool of HIV-1 substantially

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17 failed to significantly decrease the viral reservoir and the clinical results using histone

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19 deacetylase (HDAC) inhibitors such as valproic acid are controversial [10]. Therefore, the

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21 identification of potent natural or synthetic PKC agonists lacking tumour -promoter and cellular

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23 proliferative activities has opened new research avenues for the treatment of HIV-1 latency in

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25 combination with HAART. Similarly to others groups, we have shown that prostratin, a non-

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27 tumour promoter phorbol ester, is a promising lead compound to antagonize HIV-1 latency, but

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29 the relatively high concentrations required for this activity may hamper its clinical use. In this

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31 report we identify a natural jatrophone diterpene isolated from *Euphorbia hyberna*, SJ23B, which

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33 is at least one order of magnitude more potent than prostratin. SJ23B induces the internalization

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35 of the HIV-1 receptors CD4, CXCR4 and CCR5 and prevents de novo viral infection in human

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37 primary T cells at the nanomolar range. Moreover, SJ23B activates PKC and like prostratin is a

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39 non-tumorigenic compound able to activate cells where HIV is “hidden” as latent provirus. Due

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41 to their mechanisms of action, it has been proposed that short cycles of treatment with these small

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43 molecules inducing HIV reactivation combined with HAART, could contribute to a decrease of

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45 viral reservoirs. This strategy has several advantages: first, potential immunosuppression due to

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47 down regulation of CD4 and chemokine receptors will be transient and restricted to few days.

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49 Second, burst of HIV replication induced by SJ23B would be controlled not only by the action of

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3 other antiretrovirals but by SJ23B on its own. Indeed, down regulation of HIV receptors by
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5 SJ23B would contribute to inhibition of viral spread and infection of new cells. This compound
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7 therefore represents an interesting lead for the development of new anti-AIDS drugs that could be
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9 exploited as adjuvant therapy to target latent reservoirs for patients on HAART.
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Abbreviations used: PKC: Protein kinase C; HIV-1: Human immunodeficiency Virus-1; PMA: Phorbol Myristate Acetate; HAART: Highly active antiretroviral therapy; DAG: 2-diacyl-*sn*-glycerol.

ADDITIONAL FOOTNOTES. L.M.B. and N.M. contributed equally to the work

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3 **FIGURE LEGENDS**
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5 **Figure 1. Chemical structures.** Chemical structures of the diterpene isolated from *E.*
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7 *hyberna*, SJ23B, and the phorbol esters derivatives phorbol myristate acetate (PMA) and
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9 prostratin.
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14 **Figure 2. Anti-HIV activity of SJ23B.** A. MT-2 cells or PBMCs activated with CD3+IL2 for
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16 48 hours were pre treated with different concentrations of SJ23B for two hours and infected for 2
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18 additional hours with a recombinant X4 tropic virus (NL4.3-Renilla). Renilla-luciferase activity
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20 (RLUs) in cell lysates was measured 48 hours later. Results are expressed as % RLUs *versus*
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22 infection performed in the absence of drug (Untreated cells were used as control). Viability was
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24 assessed by propidium iodide method or by CellTiter Glo viability assay (Promega). B.
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26 Activated PBMCs were pre treated for 2 hours with different concentrations of SJ23B and
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28 infected with a wild type X4 tropic virus (NL4.3) or with a recombinant X4 tropic virus (NL4.3-
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30 Renilla) for 2 additional hours. Afterwards, cell culture was extensively washed with PBS and
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32 compounds were added again to the cell culture. HIV p24-gag protein was determined in culture
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34 supernatants 48 hours later. Results are expressed as % p24-gag protein *versus* infection
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36 performed in the absence of drug. Viral production in control untreated cells was 10 ng p24/ml
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38 (threshold of the test was 10 pg p24/ml). Table: Inhibitory concentration 50 (IC₅₀) and cytotoxic
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40 concentration 50 (CC₅₀) values for the panels described above were estimated using GraphPad
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42 software and Specificity index (SI) were calculated by the formula: CC₅₀/ IC₅₀. C. Activated
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44 PBMCs were treated with different concentrations of SJ23B, and left in culture for two weeks.
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46 Every 48-72 hours, culture was washed and treatment was added again. Cell viability was
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48 measured at days 2, 6, 10 and 14 by CellTiter Glo (Promega) luminescence viability assay, and
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50 results are shown as percentage of viable cells as compared to a non treated culture. Apoptosis
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3 was evaluated by Annexin V expression in cell surface at days 2, 6, 10 and 14. Results are shown
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5 as percentage of Annexin V positive cells.
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10 **Figure 3. SJ23B inhibits HIV replication at lower concentrations than prostratin.**

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12 Activated PBMCs were infected with X4 or R5 recombinant HIV carrying the Renilla-luciferase
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14 gene as reporter (NL4.3-Renilla and JR-Renilla, respectively). Cultures were pre-treated for 2
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16 hours before infection with SJ23B or Prostratin at two concentrations (0.1 and 1 μ M) and left in
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18 culture for 48 hours. Luciferase activity was measured 48 hours after infection.
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24 **Figure 4. SJ23B downregulates HIV receptors in PBMCs.**

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26 **A.** Expression of CD4, CCR5, CXCR4 receptors was analyzed in PBMCs activated with anti-
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28 CD3 + IL-2 for 48 hours. Afterwards cells were treated with either SJ23B at 0.1 or 1 μ M or
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30 PMA (0.04 μ M). Expression of receptors was measured by using specific monoclonal antibodies
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32 at different points along drug treatment (2, 5, 24 and 48 hours). Results are shown receptor mean
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34 fluorescence intensity in cell cultures (upper pannels) and as percentage of expression (lower
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36 pannels). Values are representative of at least three independent experiments. PBMCs were
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38 analyzed using forward-versus-side scatter dot plots.
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43 **B.** Expression of HLA-1 and CD69 was studied in resting PBMCs treated with either SJ23B at
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45 0.1 or 1 μ M or PMA (0.04 μ M). Expression of receptors was measured by using specific
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47 monoclonal antibodies at different points along drug treatment (2, 5, 24 and 48 hours). Results
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49 are shown and as receptor mean fluorescence intensity in cell cultures (upper pannels) and as
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51 percentage of expression (lower pannels). Values are representative of at least three independent
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53 experiments. PBMCs were analysed using forward-versus-side scatter dot plots.
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3 **Figure 5. Inhibition of HIV infection by SJ23B is related to down-regulation of viral**
4 **receptors.** PBMCs previously activated with CD3+IL-2 for 48 hours were treated with PMA
5 (0.04 μ M) or SJ23B (0.1 and 1 μ M) for five hours or left untreated. Cell cultures were then
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7 (0.04 μ M) or SJ23B (0.1 and 1 μ M) for five hours or left untreated. Cell cultures were then
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9 infected overnight with either X4 (NL4.3 Renilla), R5 (JR Renilla) tropic or VSV pseudotyped
10 (NL4.3- Δ -env-VSV-Luc) recombinant viruses (**VA**: Virus adsorption). Afterwards, PBMCs were
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12 left in culture. Non-infected (\blacktriangle) and infected but non-treated (\blacklozenge) cultures were used as
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14 controls. **A)** Expression of CD4, CXCR4 and CCR5 in activated PBMCs. CCR5, CXCR4 and
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16 CD4 expression was measured at different times during treatment (0, 4 and 7 days). Results are
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18 shown as percentage of cells expressing the receptor and are representative values of at least
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20 three independent experiments. PBMCs were analyzed using forward-versus-side scatter dot
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22 plots. **B)** Inhibition of HIV replication by SJ23B. HIV infection was measured at day 4 by
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24 luminometry in the same culture used to evaluate receptor expression and it is expressed as
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26 percentage of RLU in infected cultures as compared to non-treated cultures.
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36 **Figure 6. SJ23B antagonizes HIV-1 latency through a PKC-dependent pathway. A)**
37 Jurkat LAT-GFP cells were stimulated with either prostratin or SJ23B at the indicated
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39 concentrations for 6 hours, and next analysed by flow cytometry. Results are represented as the
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41 percentage of GFP positive cells \pm SD of three different experiments. **B)** Jurkat LAT-GFP cells
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43 were pretreated with the indicated inhibitors for 30 min at the indicated dose, and then stimulated
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45 with SJ23B (1 μ M) for 6h. The percentage of GFP+ cells was measured by flow cytometry.
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48 Results are represented as percentage of activation \pm SD compared to cells treated with agonists
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50 in the absence of the chemical inhibitors (100% activation). **C) Activation of PKC α and PKC δ**
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52 **by SJ23B.** PKC α and PKC δ activities were assayed in the presence phospholipid vesicles (20%
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3 phosphatidylserine, 80% phosphatidylcholine) and increasing concentration of SJ23B. Results
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5 are expressed as percentage of the activation observed with 1 μ M PMA (100%) and represent the
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7 mean \pm S.E. of triplicate determination in a single experiment. Two additional experiments gave
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9 similar results.
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14 **Figure 7. SJ23B induced degradation of I κ B α and strong NF- κ B binding to DNA. A)** The
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16 cells were pretreated with the inhibitors as indicated and then stimulated with SJ23B for 15 min.
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18 Total cell extracts were analysed by Western Blot with specific antibodies. **B)** Effect of SJ23B
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20 on NF- κ B-DNA binding activity. Jurkat LAT-GFP cells were incubated either with PMA (50
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22 ng/ml) or with increasing concentrations of SJ23B in the presence or absence of Go6976 (1 μ M)
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24 or Rottlerin (40 μ M) for 30 min. Nuclear extracts were then assayed by EMSA using a labelled
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26 NF- κ B oligonucleotide.
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34 **Figure 8. SJ23B translocates PKC α -GFP to the plasma membrane and synergizes with**
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36 **ionomycin to activate NF- κ B A)** Fluorescent images of CHO-K1 cells expressing the PKC α -
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38 GFP protein treated at 37 °C for 10 min with either PMA (1 μ M) or with increasing
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40 concentrations of SJ23B in the absence or the presence of ionomycin (0.5 μ M). Three additional
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42 experiments gave similar results. **B)** Jurkat LAT-GFP cells were incubated either with PMA (50
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44 ng/ml) or with increasing concentrations of SJ23B in the presence or absence of ionomycin (0.5
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46 μ M) for 30 min. Nuclear extracts were then assayed as in Figure 7.
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53 **Figure 9. Effect of SJ23B on foci formation in NIH 3T3 cells.** NIH 3T3 fibroblasts were
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55 treated either with PMA, or Prostratin, or SJ23B, or transfected with pCEFL-KZ-HA-K-Ras V12
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3 (K-RasV12) at concentrations described in the Figure. Foci formation is evident when NIH 3T3
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5 cells were transfected with KRasV12, or treated with PMA.
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10 **Figure 10. SJ23B activates HIV-1 transcription and induces NF- κ B- and Sp1-dependent**
11 **gene expression.** Resting PBMCs were transfected with different luciferase constructs under the
12 control of full-length HIV DNA (NL4.3-Luc), three tandem κ B consensus repeats (3-enhancer-
13 κ B-ConA-Luc) and Sp1 (Sp1-Luc) in the presence of SJ23B (0.1 and 1 μ M), prostratin (0.1 and
14 1 μ M) and PMA (0.04 μ M). After 48 hours, RLU were measured and results were expressed as
15 fold versus transfected and non-activated PBMCs.
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27 **Figure 11. Chemical structures of the jatrophone diterpenes used in the study and IC₅₀**
28 **values for HIV-1 reactivation in Jurkat-LAT-GFP cells.**
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Figure 2

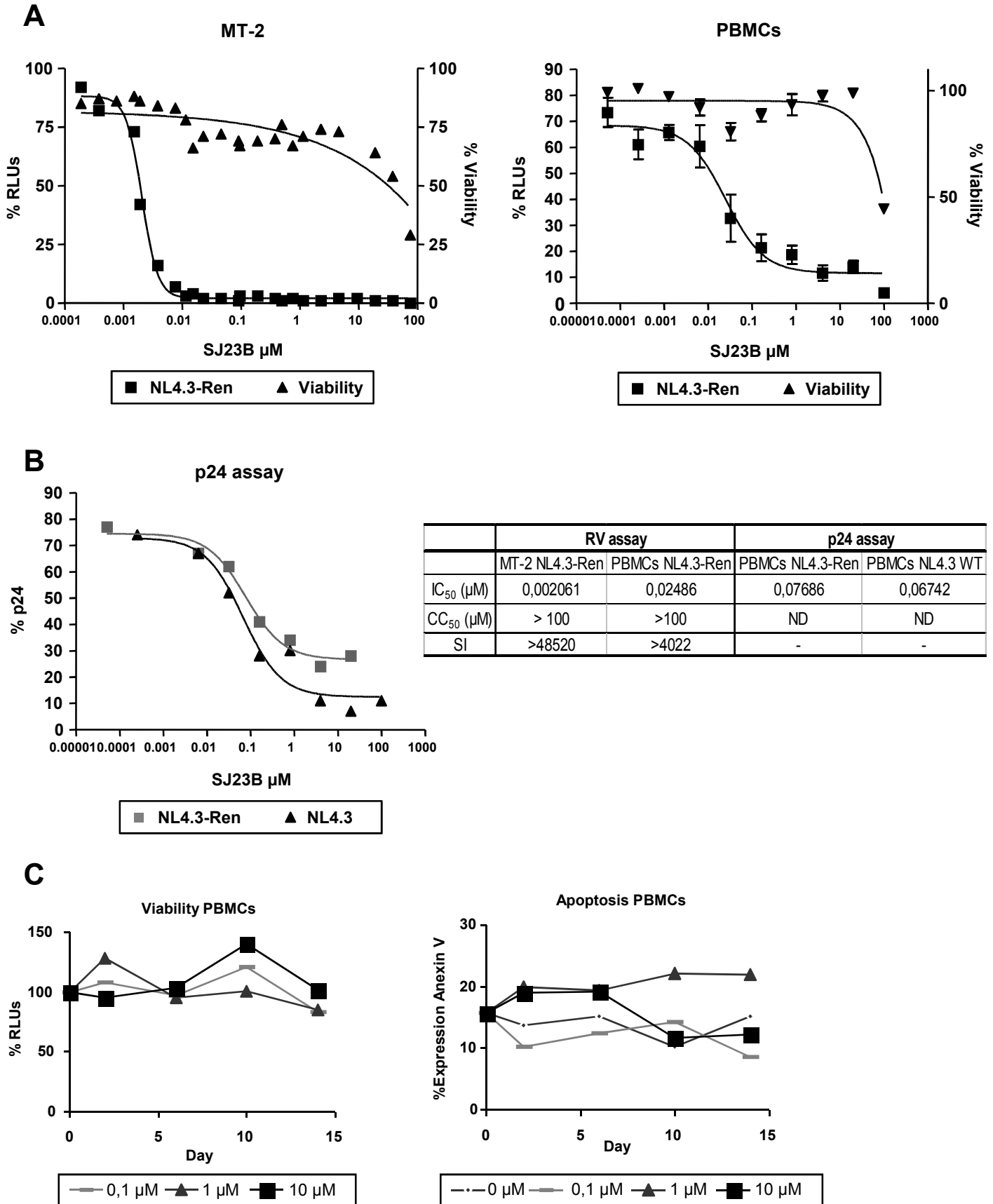


Figure 3

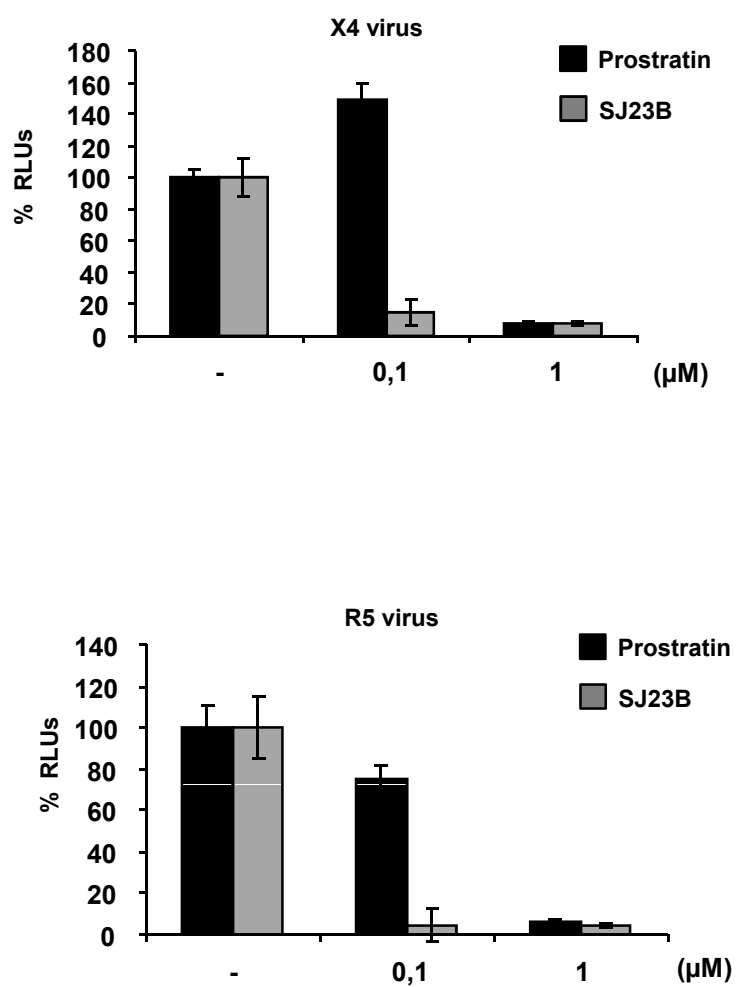


Figure 4

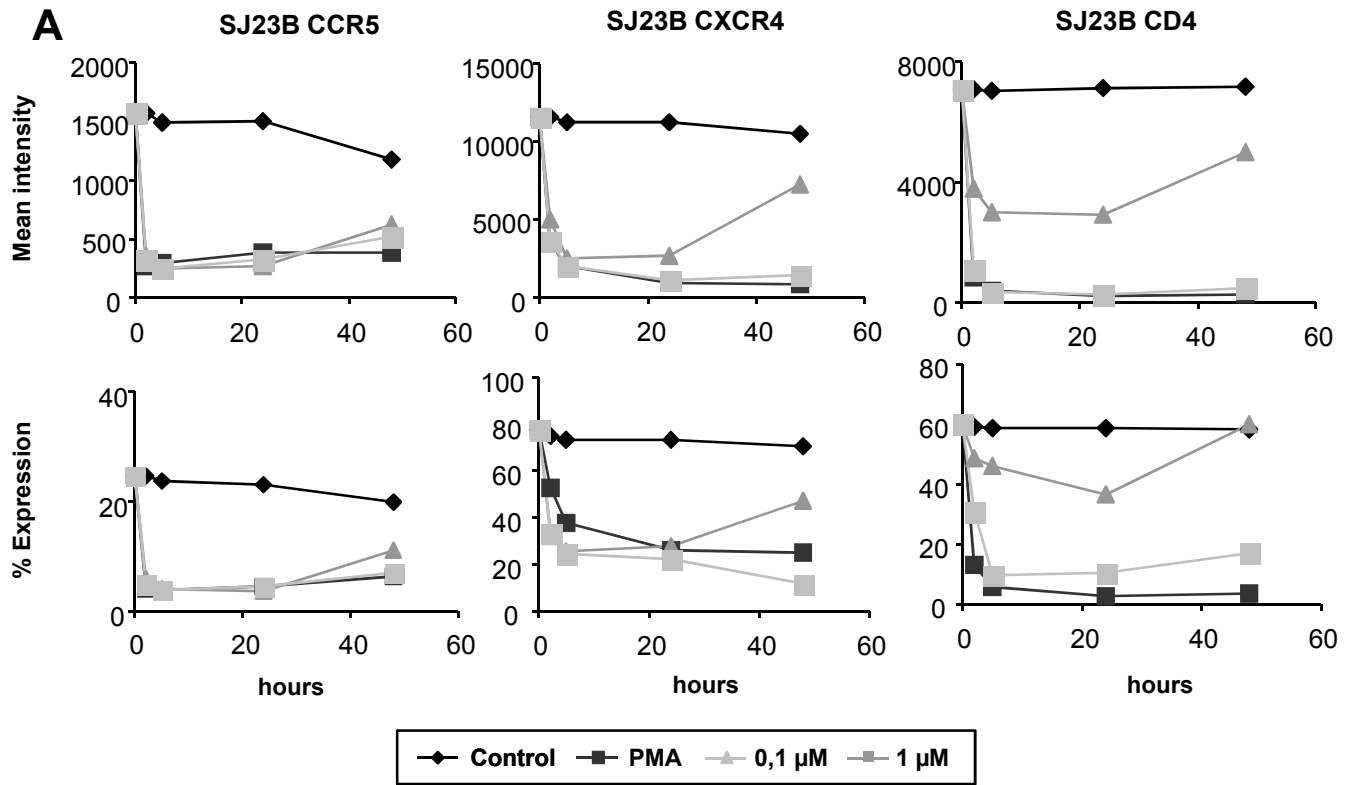
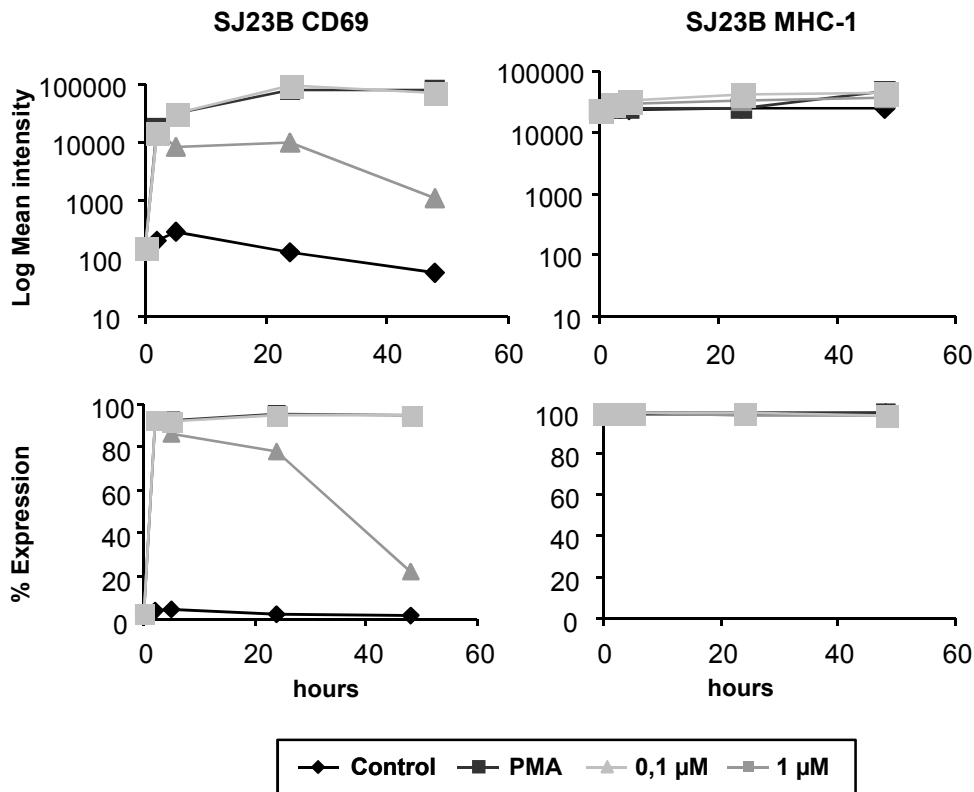
**B**

Figure 5

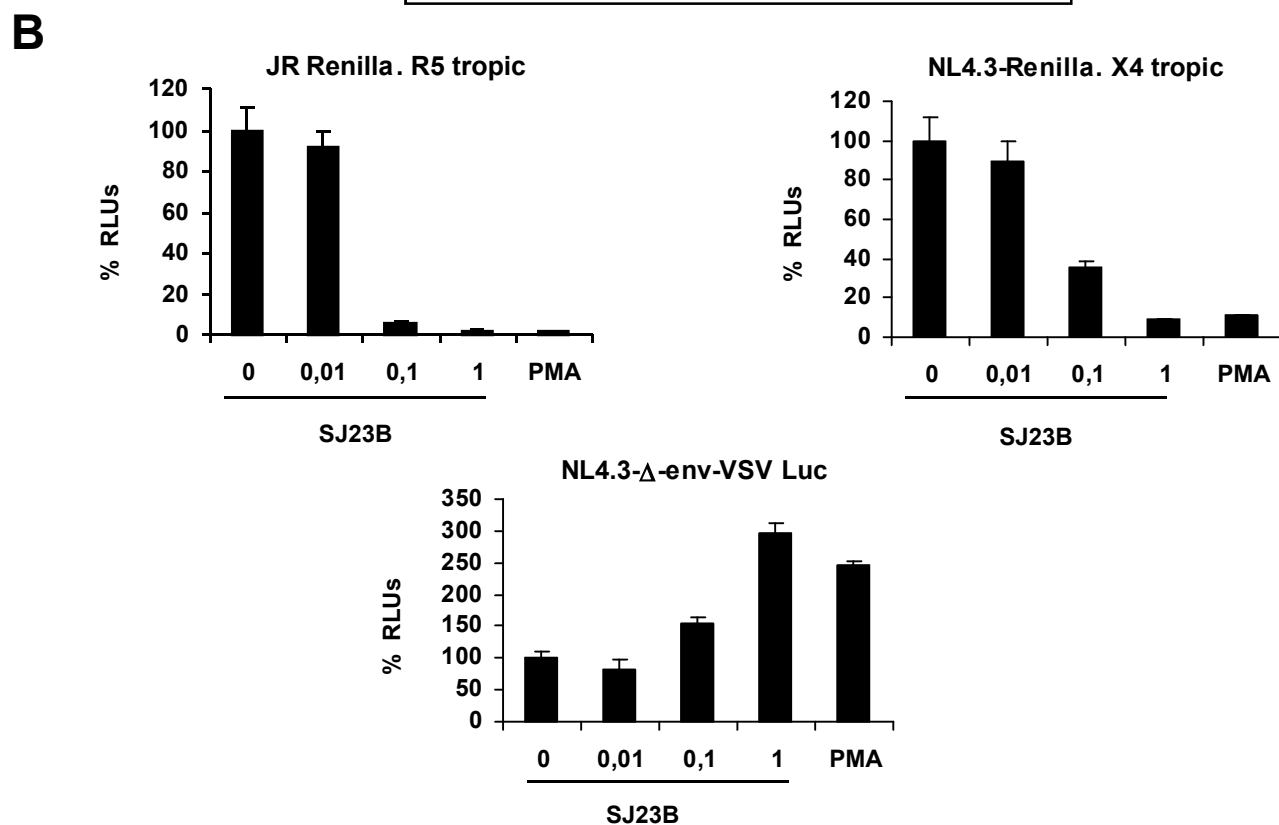
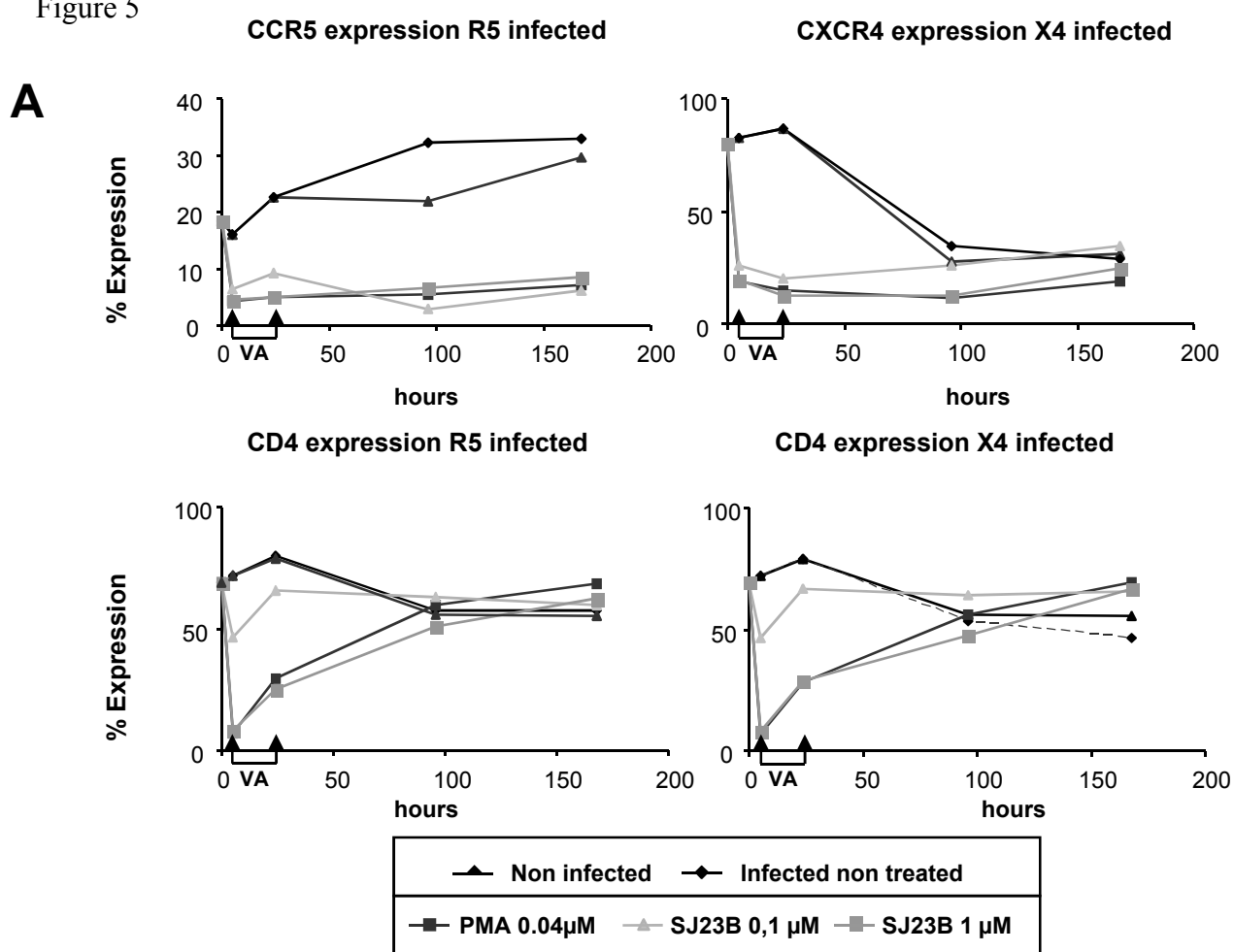


Figure 6

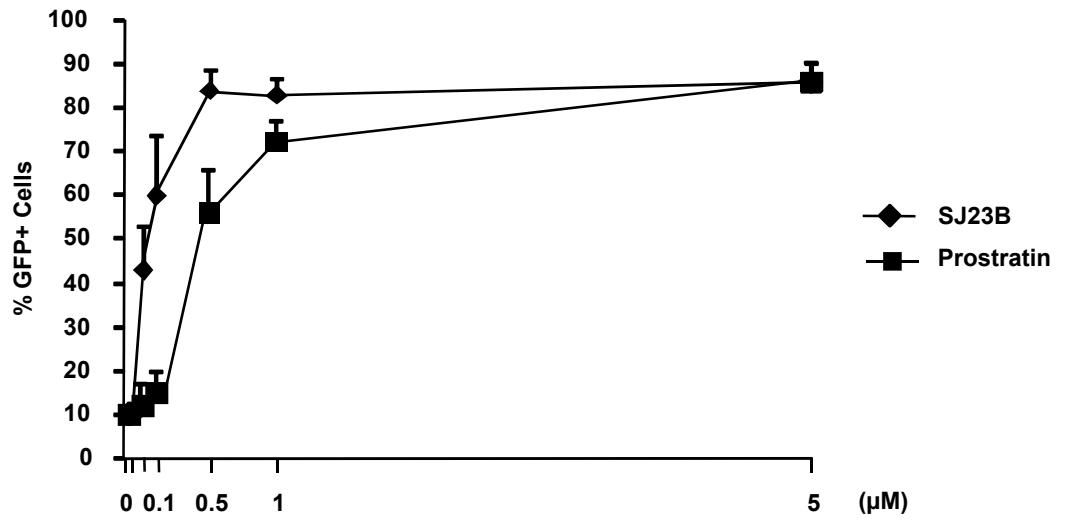
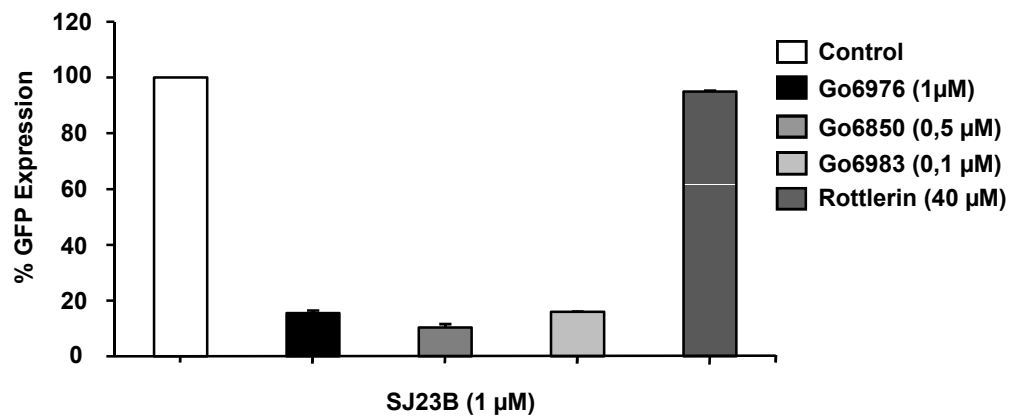
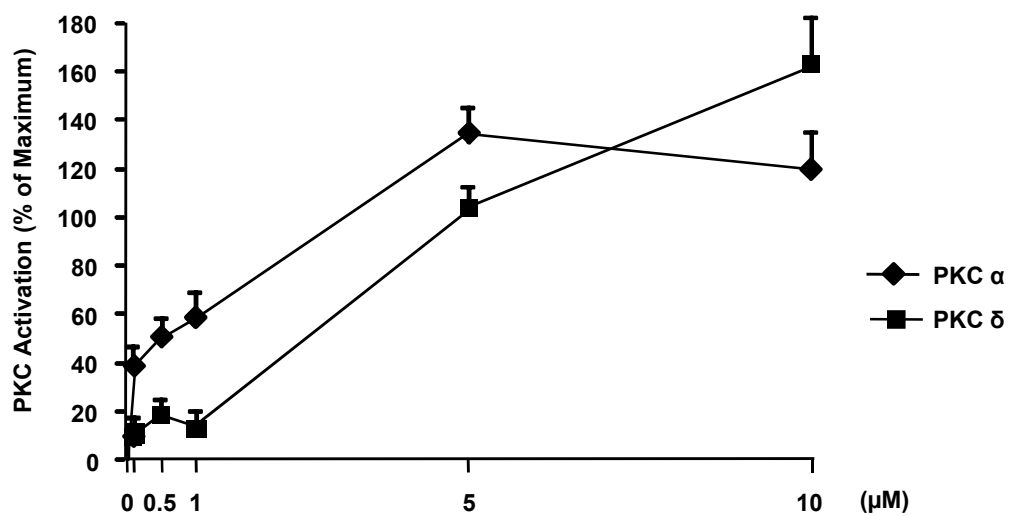
A**B****C**

Figure 7

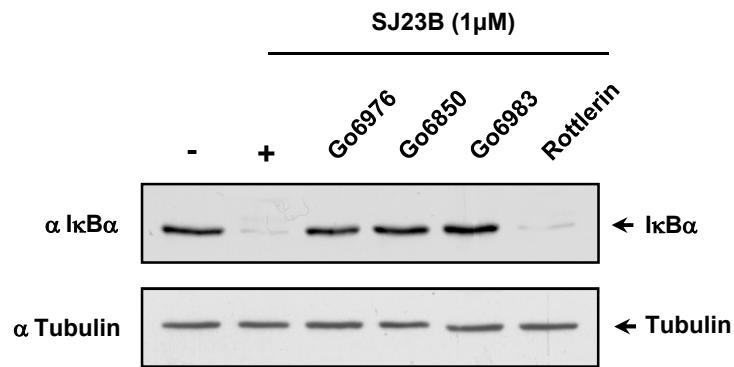
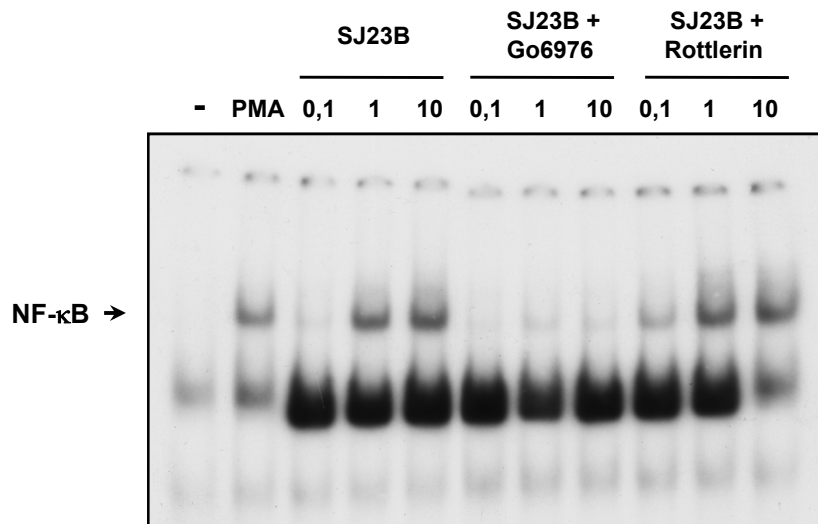
A**B**

Figure 8

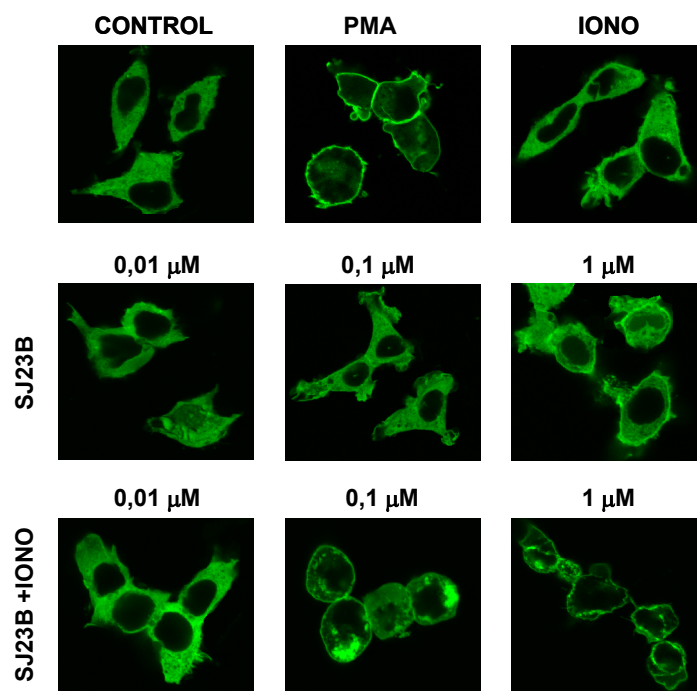
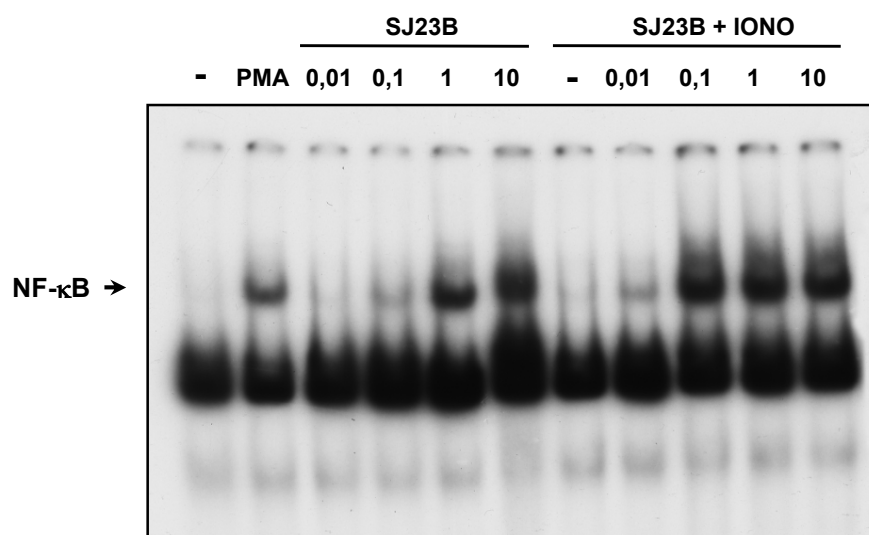
A**B**

Figure 8

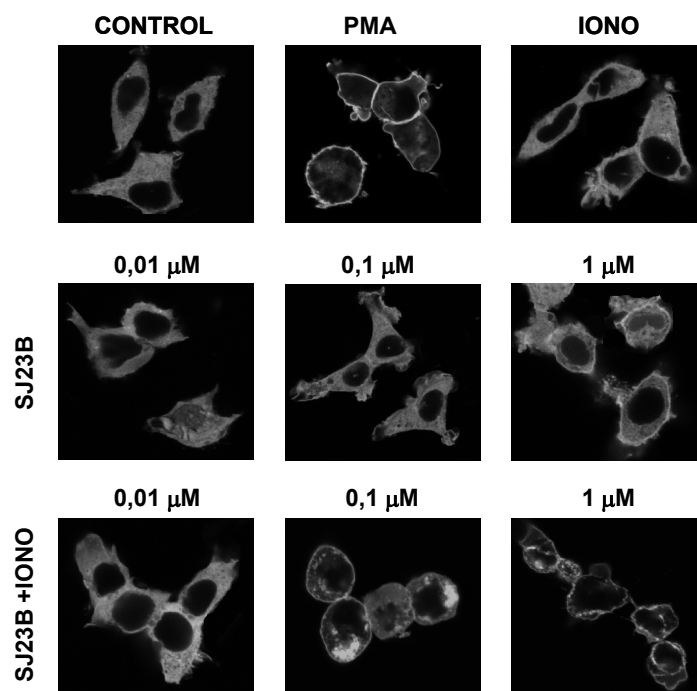
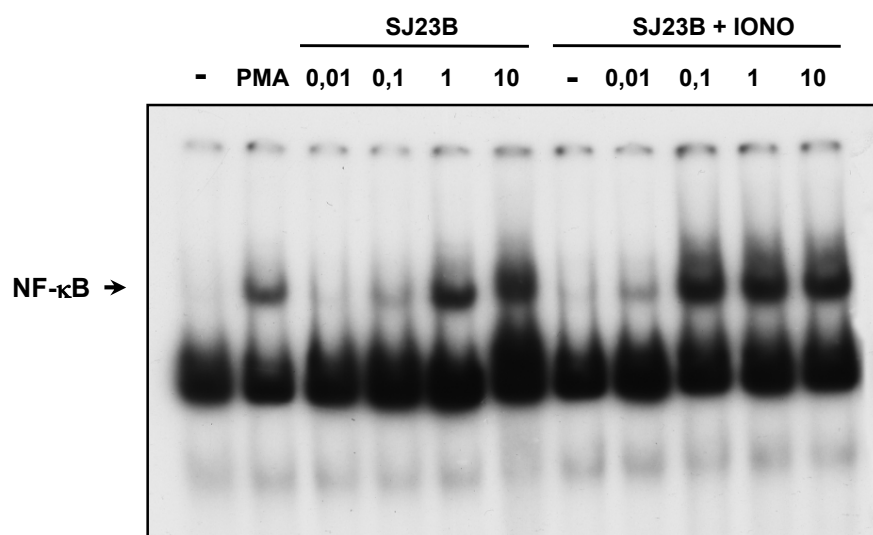
A**B**

Figure 9

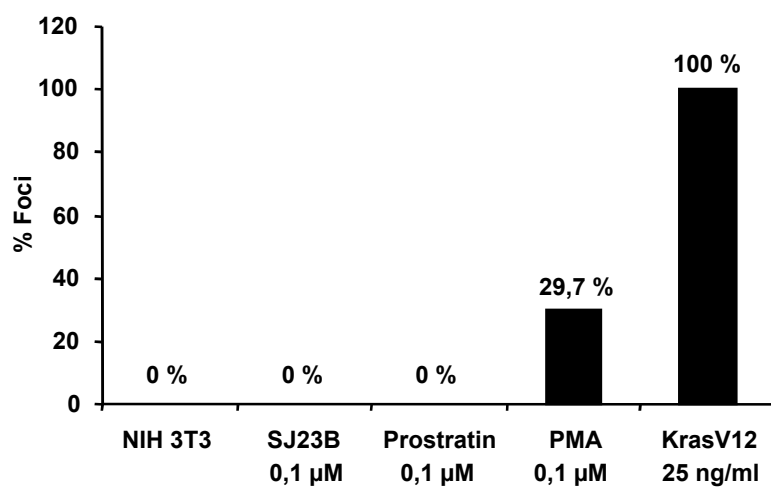
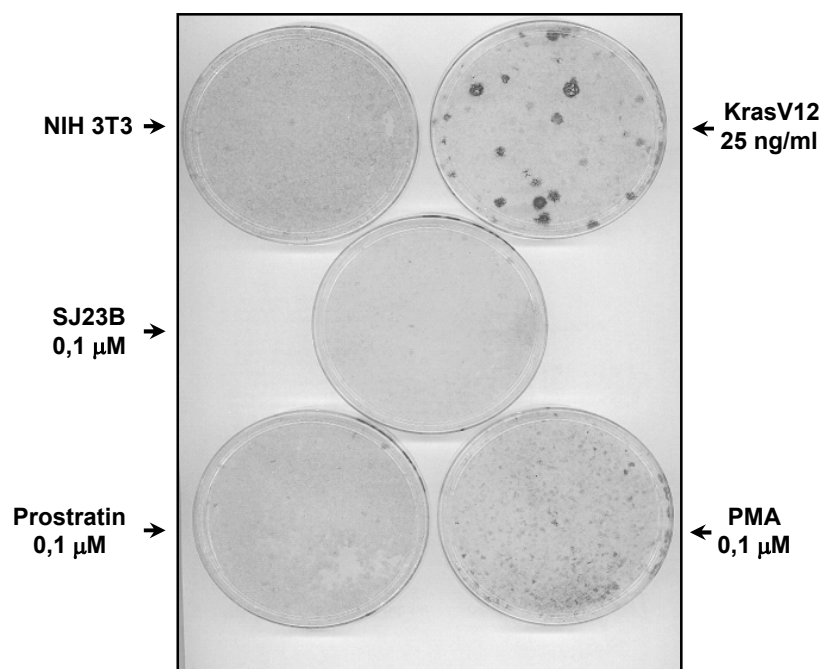


Figure 10

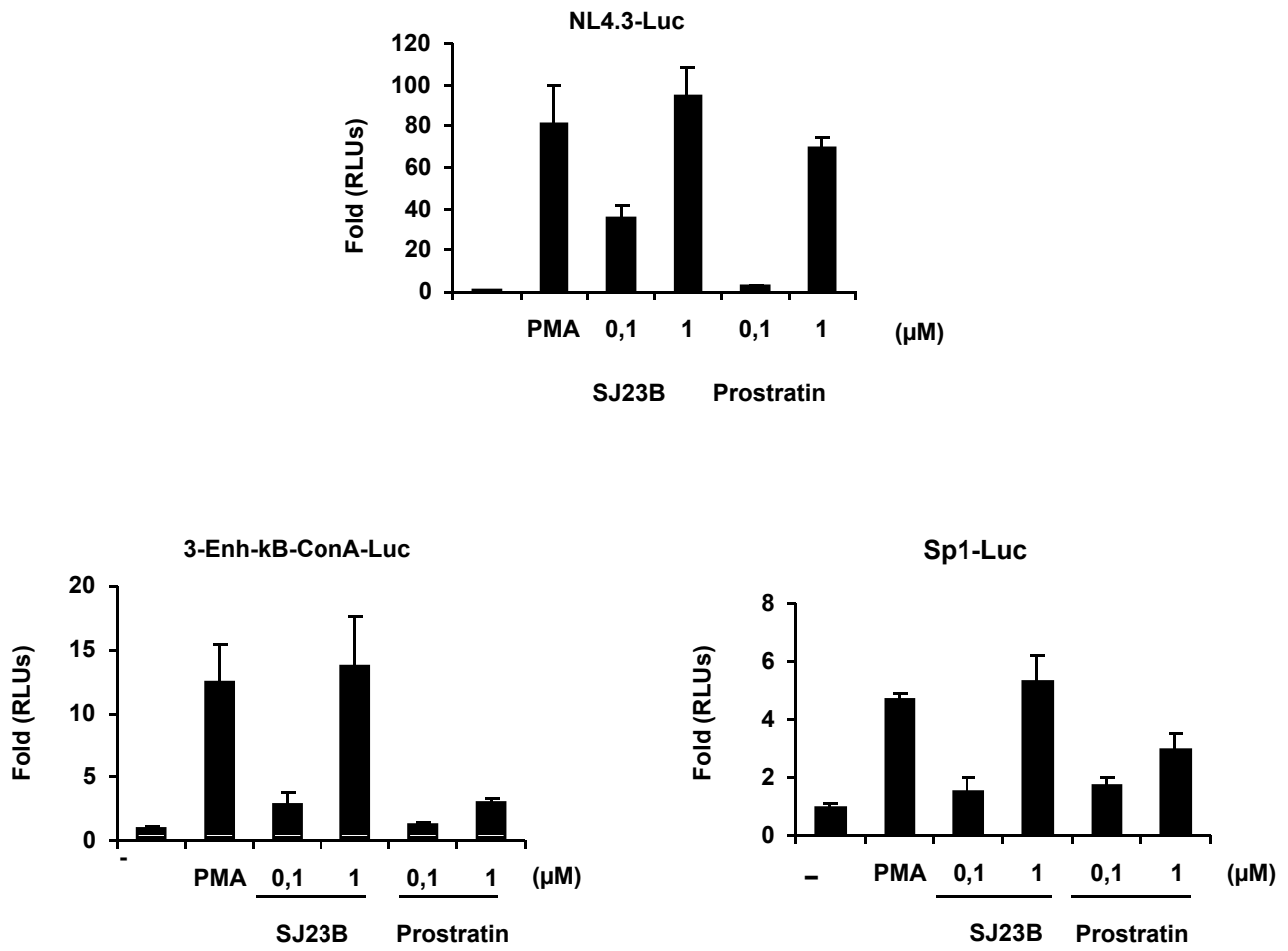
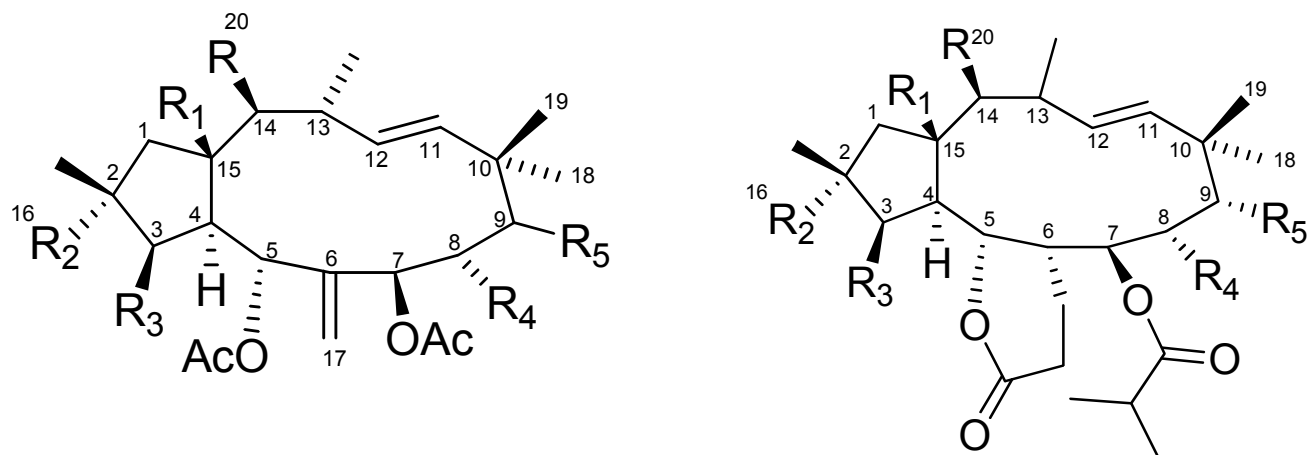


Figure 11



	R	R ₁	R ₂	R ₃	R ₄	R ₅	HIV-1 REACTIVATION EC ₅₀ (μM)
<i>SJ-13c</i>	-OAc	-OH	-H	-OBz	-H	=O	>50
<i>SJ-13d</i>	-OAc	-OH	-H	-OBzCO ₂ C ₃ H ₇	=O	>50
<i>SJ-13e</i>	-OAc	-OH	-H	-OBzOAc	=O	>50
<i>SJ-23b</i>	-OAc	-OAcOAc	-OAcOAc	=O	0,25
<i>SJ-27a</i>	-OH	-OH	-H	-OBzOAc	=O	>50
<i>SJ-29b</i>	-OAc	-OH	-H	-OBz	-HOH	>50
<i>Terracinolide C</i>	=O	-OAcOAc	-OHOAcOAc	10