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Analysis of protein kinase C theta inhibitors for the control of HIV-1 replication in human CD4+ T cells reveals an effect on retrotranscription in addition to viral transcription

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ABSTRACT

HIV-1 infection cannot be cured due to reservoirs formed early after infection. Decreasing the massive CD4+ T cell activation that occurs at the beginning of the disease would delay reservoir seeding, providing a better prognosis for patients. CD4+ T cell activation is mediated by protein kinase C (PKC) theta (θ), which is involved in T-cell proliferation, as well as NF-κB, NF-AT, and AP-1 activation. We found that PKCθ activity increased viral replication, but also that HIV-1 induced higher activation of PKCθ in infected CD4+ T cells, creating a feedback loop. Therefore, specific inhibition of PKCθ activity could contribute to control HIV-1 replication. We tested the efficacy of seven PKCθ specific inhibitors to control HIV-1 replication in CD4+ T cells and selected two of the more potent and safer: CGX1079 and CGX0471. They reduced PKCθ phosphorylation at T538 and its translocation to the plasma membrane, which correlated with decreased HIV-1 retrotranscription through partial inhibition of SAMHD1 antiviral activity, rendering lower proviral integration. CGX1079 and CGX0471 also interfered with viral transcription, which would reduce the production of new virions, as well as the subsequent spread and infection of new targets that would increase the reservoir size. CGX1079 and CGX0471 did not completely abrogate T-cell functions such as proliferation and CD8-mediated release of IFNγ in PBMCs from HIV-infected patients, thereby avoiding general immunosuppresion. Consequently, using PKCθ inhibitors as adjuvant of antiretroviral therapy in recently infected patients would decrease the pool of activated $CD4^+$ T cells, thwarting proviral integration and reducing the reservoir size.

1. Introduction

Regardless of the extraordinary progress performed in the control of the human immunodeficiency virus type 1 (HIV-1) replication by the antiretroviral therapy (ART), a cure is not yet achievable. The viral persistence is mostly due to latent reservoirs that currently represent an insurmountable obstacle for eradication. These viral reservoirs are established early in the course of the infection [1-5]. The preferential targets are the activated CD4+ T cells from the gut-associated lymphoid tissue (GALT) [6,7], where the virus performs a massive replication during the first weeks of the disease. Recent findings suggest that the seeding of the viral reservoir in mucosa and lymphoid tissues may occur within days after the infection, even before the viraemia is detectable [5]. HIV-1 may then remain hidden in specific CD4 subsets, in a latent state [8-10], providing a long-lived viral reservoir that is insensitive to host immune response and effective ART. This reservoir is not static and can be increased throughout the illness by different mechanisms such as low level ongoing replication and infection of new targets or homeostatic proliferation of latently-infected CD4+ T cells [8,11]. The size of the reservoir is proportional to disease progression and influences prognosis [12,13]. Consequently, early initiation of ART would reduce the size of the reservoir [14-16], providing a better scenario for the patients.

T cell activation promotes cell functions necessary for HIV-1 infection and replication, such as CCR5 receptor expression, synthesis of dNTPs required for full genome retrotranscription, increased ATP levels necessary for the transport of the pre-integration complex to the nucleus [17-20], and activation of transcription factors involved in HIV-1 gene expression such as NF-κB, NF-AT, and AP-1 [21,22]. T-cell activation also induces apoptosis in infected cells, which is mostly responsible for the massive CD4+ T cell depletion characteristic of HIV-1 infection [23]. Accordingly, the

level of CD4+ T cell activation at early stages of the illness correlates with both the level of destruction of the immune system and the size of the reservoir.

One key regulator of cell cycle progression is SAMHD1 (sterile alpha motif domain and HD domain-containing protein 1), a major restriction factor that blocks early reverse transcription of the HIV-1 genome and induces resistance to the infection in macrophages, dendritic cells, and resting CD4+ T cells [24]. When active, SAMHD1 depletes the intracellular dNTP pool, reducing the susceptibility of resting CD4+ T cells to infection, which can be counteracted in both HIV-1 [25] and simian immunodeficiency virus (SIV) [26] by the addition of dNTPs. An alternative mechanism based on an RNase activity that leads to degradation of viral RNA has also been proposed for SAMHD1 [27]. Phosphorylation of SAMHD1 at T592 by cyclin A2/Cdk1 negatively regulates its dNTP triphosphohydrolase and RNase activities, allowing HIV-1 replication [27,28].

Several studies have shown that, in addition to reducing reservoir size, early initiation of ART suppresses viral replication and spreading, restores $CD4^+$ T cell counts, preserves HIV-1-specific T-cell responses, and controls plasma viral load after ART is discontinued [29-39]. Early use of ART would also be expected to reduce the massive immune activation induced by HIV-1 infection [40,41]. The addition of an agent that blocks T cell activation, leaving T cells refractory to HIV-1 proviral integration, might be a beneficial strategy [42]. Studies testing immunosuppressive agents such as cyclosporin A with ART at very early stages of the infection showed a beneficial impact on the long-term course of the disease [34,42-44]. However, due to the potential risk of lymphoproliferative disorders, general immunosuppressors cannot be used in long-term treatment [45]. Accordingly, a short-term, more specific immunomodulation treatment would be preferred.

Protein kinase C (PKC) theta (θ) is essential for most T cell activation pathways. It is selectively expressed in T cells but not in B cells, neutrophils, monocytes, or macrophages [46]. PKCθ is activated through T-cell receptor (TCR)/CD28 engagement at the immunological synapse [47], initiating a cascade of events that culminates in the activation of NF-κB, NF-AT, and AP-1 [22]. The kinase activity is mediated by phosphorylation at several residues [48], but phosphorylation at threonine 538 (T538) by MAP4K3/GLK and subsequent translocation to the plasma membrane is widely used as a surrogate marker for kinase activation as is most critical for the catalytic activity [22]. During T-cell anergy, PKCθ activity is controlled by the E3 ubiquitin-protein ligase Cbl-b [49], which promotes TCR down-regulation [50], regulates CD28-dependence of T cell activation [51], and represses the activation of NF-κB, NF-AT, and AP-1 [52-54]. Reciprocally, during T-cell activation, PKCθ directly regulates the ubiquitination and degradation of Cbl-b, causing a productive immune response [55].

We reported previously that blocking PKCθ selectively with rottlerin - a dose-dependent, cell-permeable inhibitor of PKCθ - or mRNA interference reduces HIV-1 replication in CD4+ T cells, inducing a refractory state to HIV-1 infection [56]. Specific inhibition of PKCθ would be expected to affect mainly T cells, without causing general immunosuppression. As rottlerin shows a narrow therapeutic window, and would be problematic as a therapeutic, we analyzed the effect on HIV-1 replication of a panel of seven selective PKCθ inhibitors in order to find a more suitable candidate and further explore the mechanism by which PKCθ contributes to HIV-1 replication. We selected two of the more potent and safer compounds and demonstrated that they acted at two different levels: first, reducing retrotranscription by interfering with SAMHD1 phosphorylation, which as a consequence rendered decreased proviral integration; and second, blocking viral transcription by inhibiting NF-κB, NF-AT, and AP-1

transactivation. The use of these inhibitors in combination with ART in newly infected patients would avoid high peaks of viral replication, reducing the spread of the infection and massive T-cell destruction, preserving the function of the immune system, and decreasing the reservoir seeding.

2. Materials and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy donors by centrifugation through a Ficoll-Hypaque gradient (GE Healthcare, Milwaukee, WI). Jurkat E6-1 cells were obtained from the NIH AIDS Reagent Program [57]. Human CD4+ T lymphocytes were isolated from PBMCs by using CD4+ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. All cell types were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2mM L-glutamine, 100μg/ml streptomycin, 100U/ml penicillin (Biowhittaker, Walkersville, MD). PBMCs were activated by treatment with 5μg/ml PHA (Sigma-Aldrich, St. Louis, MO) and 300 units/ml IL-2 (Chiron, Emeryville, CA).

PBMCs from HIV-infected patients were provided by Hospital Clinic (Barcelona, Spain). Patients were chronic, asymptomatic, treated HIV-1 infected individuals with a baseline CD4+ T lymphocyte counts > 500 cells/mm^3 and plasma viral loads ranging from 50-10,000 HIV-1 RNA copies/ml. All individuals gave informed written consent and this study was approved by the Institutional Ethical Committee board of Hospital Clinic (Barcelona, Spain).

2.2. Reagents and antibodies

Compound BIX02671 (compound 20) was kindly provided by Dr. Maryanne Brown (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT) [58]. Compound PF-05263557 was kindly provided by Dr. Nathan Tumey (Pfizer Research, Pearl River, NY) [59]. Compounds CGX1039, CGX1079, and CGX0471 were kindly provided by Dr. Joel Hedgpeth and Dr. John Swindle (CompleGen, Inc., Seattle, WA) [60] and

chemical structures can be found in US Patents 8436025 and 8664277. Compounds VE-14-26 and VE-14-27 were kindly provided by Dr. Juan-Miguel Jimenez (Vertex Pharmaceuticals (Europe) Limited, Oxford, UK) [61]. The pan-PKC inhibitor sotrastaurin/AEB071 was kindly provided by Dr. Eva López-Martín (Novartis, Basel, Switzerland) [62]. A description of the PKC inhibitory activity of these compounds is described in Table 1. Phytohemagglutinin (PHA) (Sigma-Aldrich) was used at 5μg/ml. 5-phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) was used at 25ng/ml. Stromal cell-derived factor-1 alpha (SDF-1α)/CXCL12 was used at 50 nM and was kindly provided by Dr. Françoise Baleux (Institut Pasteur, Paris, France).

A monoclonal antibody against HIV-1 Tat (aa 2–9) was obtained from Advanced Biotechnologies Inc. (Columbia, MD). Polyclonal antibody against phospho-PKCθ (Thr538) was obtained from Cell Signaling Technology (Danvers, MA). Antibody against SAMHD1 phosphorylated at T592 for immunoblotting was kindly provided by Dr. Monsef Benkirane and Dr. Benjamin Descours (Institute of Human Genetics, Montpellier, France). Antibody against total SAMHD1 was purchased from Bethyl Laboratories (Montgomery, TX). Monoclonal antibodies against Cdc2/Cdk1 (clone P0H1) and Cyclin A (clone BF683) were obtained from Cell Signaling Technology. A monoclonal antibody against β-actin (clone AC-15) was obtained from Sigma-Aldrich. Secondary antibodies conjugated to Alexa 488 and Alexa 546 were purchased from Molecular Probes (Eugene, OR). Secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from GE Healthcare. Antibodies against CD4 conjugated with PerCP, the chemokine (CXC motif) receptor 4 (CXCR4) conjugated with phycoerythrin, and the chemokine (C-C Motif) receptor 5 (CCR5) conjugated with FITC were purchased from BD Biosciences (San Jose, CA). Phalloidin conjugated with

FITC was purchased from Life Technologies (Carlsbad, CA). 4′,6-diamidino-2-phenylindole (Dapi) was obtained from Sigma-Aldrich.

2.3. Vectors

Vector pPKCθ-wt contains full-length cDNA of PKCθ; vector pPKCθ-A/E contains cDNA encoding a constitutively active PKCθ mutant by a substitution of E for A in its inhibitory pseudosubstrate sequences, position 148; and vector pPKCθ-K/R contains cDNA encoding a PKCθ dominant-negative (kinase inactive) mutant by the substitution of R for K in position 409 [63]. These three vectors were kindly provided by Dr. Julián Pardo (Science Faculty, Zaragoza University, Spain). Vector pNL4-3 wild-type (wt) that contained the HIV-1 complete genome and induced an infectious progeny after transfection was kindly provided by Dr M.A. Martin [64]. Vector pNL4.3-Renilla was obtained by replacing *nef* gene of HIV-1 proviral clone pNL4.3 with Renilla luciferase gene, as previously described [65]. Full-length HIV-1 DNA not expressing HIV envelope (pNL4.3-Luc-R_E_) was obtained from AIDS Research and Reference Reagent program (NIAID, National Institutes of Health, MD, USA). Vector pcDNA-VSV contained DNA for vesicular stomatitis virus (VSV) G glycoprotein cloned in pcDNA3.1 plasmid (Life Technologies) [66]. pLTR-LUC vector containing the luciferase (LUC) reporter gene under the control of HIV-1 LTR U3R region (LAI strain) was described previously [67]. pκB-LUC vector that contains a luciferase gene under the control of three -κB consensus motifs of the immunoglobulin κ-chain promoter was described previously [68]. The expression construct pNFAT-LUC containing three tandem copies of the distal NFAT-binding site of the IL-2 gene promoter coupled to the IL-2 minimal promoter was kindly provided by Dr Juan Miguel Redondo (National Center for Cardiovascular Research, Madrid, Spain) [69]. pAP1-LUC vector driven by

the −36 to +37 rat prolactin minimal promoter under the control of four tandem copies of the 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element consensus motif TGACTCA was also provided by Dr Juan Miguel Redondo [70].

2.4. Transient transfection and vectors

Jurkat cells were transiently transfected with an Easyjet Plus Electroporator (Equibio, Middlesex, UK). In brief, 10^7 Jurkat were collected in 350μl of RPMI without supplement and mixed with 1μg/10^6 cells of plasmid DNA. Cells were transfected in a cuvette with 4mm electrode gap (EquiBio), at 280V, 1500μF and maximum resistance. After transfection, cells were incubated in supplemental RPMI for 18h, at 37ºC. Luciferase and Renilla activities were assayed with Luciferase Assay System (Promega Ibérica, Madrid, Spain), according to manufacturer's instructions. Relative light units (RLUs) were measured in supernatants with a Sirius luminometer (Berthold Detection Systems, Oak Ridge, TN). In all transfections, data were normalized according to co-transfection with pEYFP-C1 vector, which was used as control of transfection efficiency, and to cell viability that was determined by flow cytometry. RLUs were also normalized by measuring protein concentration [71] in a microplate reader Sunrise (Tecan Group Ltd., Männedorf, Switzerland).

2.5. Immunofluorescence assay

Cells were stained *in vivo* with cholera toxin B subunit conjugated with FITC (Sigma-Aldrich), adhered on PolyPrep slides (Sigma-Aldrich), fixed with 2% paraformaldehyde in PBS1X and permeabilized as previously described [72]. Cells were then stained with a specific primary antibody against PKCθ phosphorylated at Thr^{538} and a secondary antibody conjugated to Alexa 546 or with an antibody against

Tat and a secondary antibody conjugated to Alexa 488. Nuclei were stained with Dapi. Images were obtained with a Leica DMI 4000B Inverted Microscope (Leica Microsystems, Barcelona, Spain). The intensity mean per pixel was calculated in all images using LAS AF Lite software (Leica Microsystems) and values were represented in bar diagrams showing statistical significance.

2.6. Immunoblotting assays

Whole, cytosolic, and membrane protein fractions were obtained as described previously [73,74]. Protein concentration was determined by Bradford method [71]. Forty micrograms of protein extracts from each fraction were fractionated by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) and transferred onto Hybond-ECL nitrocellulose paper (GE Healthcare). After blocking and hybridization, proteins were detected with SuperSignal West Pico/Femto Chemiluminescent Substrate (Pierce, Rockford, IL). Images were acquired in a Bio-Rad Geldoc 2000 (Bio-Rad Laboratories, Madrid, Spain) and analyzed by densitometry with Quantity One software (Bio-Rad). Values were represented as bar diagrams showing the statistical significance.

2.7. HIV-1 infection

Infectious supernatants were obtained from calcium phosphate transfection of 293T cells with plasmids pNL4-3_wt or pNL4-3_Renilla. VSV-pseudotyped ΔEnv-NL4.3-Luc virus was obtained by co-transfection of pNL4.3-Luc-R_E_ and pcDNA-VSV in HEK293T cells as previously described [75]. Classic antiviral assays were performed by pre-treating PBMCs with PKCθ inhibitors for 30 minutes before or after adding PHA/IL-2 for two days. Cells were then infected by spinoculation for 30 minutes with NL4-3_Renilla strain at gently rotation, room temperature. After centrifugation at 600xg for 30min at 25°C and extensive washing with PBS1X, each

PKCθ inhibitor was added again and cells were left in culture for 2-7 days. Renilla or luciferase activity was quantified in the cell lysates as described above.

The half maximal inhibitory concentration (IC_{50}) was determined by incubating PBMCs activated with PHA/IL-2 for 2 days in a 96-well microtiter plate in the presence of 0.5ng p24/well of NL4-3_Renilla, as well as increasing concentrations of each PKCθ inhibitor. Production of Renilla (RLUs), corresponding to HIV-1 replication, was measured 48h post-infection. IC_{50} and the half maximal cytotoxic concentration (CC_{50}) were calculated using GraphPad Prism software (GraphPad, San Diego, CA) (sigmoidal dose–response formula). Results were expressed using the selectivity index (SI = CC_{50}/IC_{50}).

2.8. Cell viability and proliferation

Cell viability was determined with the CellTiter-Glo® Luminescent Cell Viability Assay (Promega), following manufacturer's instructions. Cell lysates were analyzed in an Orion Microplate Luminometer with Simplicity software (Berthold Detection Systems). Cell proliferation was measured by flow cytometry after staining with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) as previously described [76]. Briefly, cells were labeled with CFSE 2μM, washed, and then resuspended in medium and cultured in 96-well round-bottomed plates in the absence or presence of staphylococcal enterotoxin A (SEA) for 6 days. Samples were acquired with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and data analysis was performed with FlowJo software v7.2.5 (TreeStar, Ashland, OR) using uses non-linear curve-fitting techniques. The number of generations (Gn) was fixed at eight peaks, each including the corresponding number of events for each generation.

2.9. Enzyme-linked immunospot (ELISPOT) assay for IFN-γ release

Ex vivo measurement of antigen-specific IFN-γ production from CD8 T cells was performed by ELISPOT as previously described [77,78]. Briefly, 1-2x10^5 PBMCs from HIV-infected patients were incubated for 18h in 96-well plates coated with anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden). Different HLA class I-restricted synthetic peptides from Gag, Pol, Env and Nef, as well as peptide pools (15-mer peptides overlapping by 11) spanning the entire HIV-1 consensus B Gag proteins sequences (NIH AIDS Reagent Program), were used. A synthetic HLA*02 restricted-CMV pp65 peptide was also tested. RPMI/10%FCS was used as negative control. A pool of MHC class I-restricted T cell epitopes from human cytomegalovirus, Epstein-Barr virus and influenza virus (CEF) (Mabtech) and PHA were used as positive controls. Cells were lysed, incubated with biotin-labelled, anti-IFN-γ mAb 7-B6-1 (Mabtech) and then with streptavidin-alkaline phosphatase (Mabtech) to visualize the spot forming foci (IFN-γ-secreting cells). After adding a chromogenic alkaline phosphatase conjugated substrate (BioRad), the spot forming cells (SFC) were counted using an AID ELISPOT reader (Autoimmun Diagnostika GmbH, Strassberg, Germany). Results were considered positive if the number of SFC/10^6 PBMCs in stimulated wells was 2-fold higher than that in unstimulated control wells, and if there were at least 50 SFC/10^6 PBMCs after background subtraction.

2.10. F-actin polymerization assays

Cells cultured during 15 seconds at 37ºC in the absence or presence of SDF-1a/CXCL12 were fixed and permeabilized with 2% PFA and 0.1% Triton X-100, respectively. Phalloidin-FITC conjugated (6 U/ml) was added for 15 min at 37ºC. Phalloidin binds at the interface between F-actin fibers, avoiding its depolymerization. The amount of polymerized F-actin was determined by flow cytometry with a

FACSCalibur flow cytometer (Becton Dickinson) and FlowJo software v7.2.5 (TreeStar).

2.11. Quantification of early and late retrotranscription by TaqMan qPCR

Five to seven hours after infection, DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen Iberia, Madrid, Spain) and quantified at 260/280nm using a Nanodrop 2000C (Thermo Scientific, Wilmington, DE). Early and late retrotranscription (RT) were assessed by qPCR as described [79] Konig et al (2008). Briefly, 50-100ng DNA were mixed with 1μM forward and reverse primers for early RT (MA.pr-243 (for) 5'-GTGCCCGTCTGTTGTGTGAC-3'; MA.pr-244 (rev) 5'-GGCGCCACTGCTAGAGATTT-3') and late RT (MA.pr-245 (for) 5'-TGTGTGCCCGTCTGTTGTGT-3'; MA.pr-246 (rev) 5'-GAGTCCTGCGTCGAGAGATC-3'), 0.2μM FAM/ZEN/Iowa Black probe (Integrated DNA Technologies, Coralville, IA) (early RT: MA.pr-275 5'-CTAGAGATCCCTCAGACCCTTTTAGTCAGTGTGG-3'; late RT: MA.pr-276 5'-CAGTGGCGCCCGAACAGGGA-3'), and 1xTaqMan Universal Master Mix II (Life Technologies) in a final volume of 16μl. qPCR was performed in triplicate in a StepOne Real-Time PCR system (Life Technologies) using standard cycling conditions. Serial dilutions of genomic DNA from 8E5 cell line, which contain a single integrated copy of HIV-1 [80], were used as standard curve. *ccr5* gene was used as endogenous control.

2.12. Quantification of proviral integration by TaqMan qPCR

Proviral integrated DNA was quantified in whole genomic acid from HIV-infected CD4+ T cells by using nested Alu-LTR PCR as previously described [81,82] using a StepOne Real-Time PCR System (Life Technologies). In brief, a first

conventional PCR was performed using oligonucleotides against Alu sequence and the HIV-1 LTR, with the following conditions: 95ºC, 8 min; 12 cycles: 95ºC, 1 min; 60ºC, 1 min; 72ºC, 10 min; 1 cycle: 72ºC, 15 min. Then, a second qPCR was performed using TaqMan probes with FAM/ZEN/Iowa Black (Integrated DNA Technologies) and TaqMan Master Mix (Life Technologies). *ccr5* gene was used as endogenous control.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad). Comparisons between groups were made using two-way analysis of variance (ANOVA) with Bonferroni post-test analysis to describe the statistical differences among groups. The P values (p) <0.05 were considered statistically significant in all comparisons and were represented as *, **, ***, or **** for $p<0.05$, $p<0.01$, $p<0.001$, or $p<0.0001$, respectively.

3. Results

3.1. Evaluation of selective PKCθ inhibitors for control of HIV replication versus cytotoxicity in CD4+ T cells

PBMCs isolated from healthy donors, activated with PHA and IL-2 for 48 hours, were pre-treated for 30 minutes in a 96-well microtiter plate with each of seven different selective inhibitors of PKCθ (BIX02671, PF-05263557, CGX1039, CGX1079, CGX0471, VE-14-26 or VE-14-27) or with the pan-PKC inhibitor sotrastaurin/AEB071, at serial concentrations (0-100 μM). Sotrastaurin/AEB071 is a potent inhibitor of all PKC isoforms involved in T cell function (PKCα, PKCβ, PKCδ, PKCε, and PKCθ) [83] and was used as control for non-specific inhibition of PKCθ. Cells were then incubated for 48 hours with 0.1ng p24 of recombinant HIV-1 NL4-3_Renilla strain per culture well, in the presence of IL-2. All inhibitors were added to the culture medium only at the beginning of the experiment. Production of Renilla (RLUs), which correlated with HIV-1 replication [65], was measured to calculate IC_{50} (Fig. 1A, left line graph). Cell viability was evaluated in cells treated in parallel and CC_{50} was determined for each inhibitor (Fig. 1A, right line graph). The selectivity index ($SI = CC_{50}/IC_{50}$) was calculated to identify PKCθ inhibitors with high therapeutic index, giving maximum antiviral activity with minimal cytotoxicity. All inhibitors with $SI > 4.0$ were considered for further analysis. BIX02671 and VE-14-26 showed $SI < 4.0$ (2.74 and 3.0, respectively), whereas PF-05263557 showed the highest SI (17.63). VE-14-27 showed high cytotoxicity, with very low SI (0.96; $IC_{50} = 21.81$ μM; $CC_{50} = 20.98$ μM) (data not included in Fig. 1A). It was discarded from the study.

As HIV-1 induces the formation of syncytia in MT-2 cells, a widely used cell line for the study of HIV-1 infection, the presence of syncytia was analyzed in these

cells 5 days after the infection with NL4-3_wt strain in the presence or not of PKCθ inhibitors with high SI (CGX1079, CGX0471, CGX1039, and PF-05263557) (Fig. 1B). CGX1079 and CGX0471 completely impeded the formation of syncytia, whereas CGX1039 was less effective and some syncytia appeared at the end of the 5-days culture.

PHA/IL-2-activated PBMCs infected with NL4-3_Renilla for 7 days and incubated with Rottlerin, PF-05263557, CGX1079 and CGX0471, which were added to the culture medium only at the beginning of the experiment, showed that all compounds except PF-05263557 retained their inhibitory capacity after 7 days in culture. PF-05263557 seemed then to be degraded after more than 3 days in culture (Fig. 1C). According to these results, CGX1079 and CGX0471 were selected for further study.

3.2. Effect of CGX1079 and CGX0471 on T cell proliferation

We evaluated the effect of CGX1079 and CGX0471 on PKCθ-dependent T-cell functions such as proliferation at the effective concentration to inhibit HIV-1 replication (10 μM). PBMCs were left untreated or primed with staphylococcal enterotoxin A (SEA) for 6 days. The effect of CGX1079 and CGX0471 on T-cell proliferation was analyzed by labeling with CFSE and subsequent analysis by flow cytometry. Sotrastaurin/AEB071 1 μM was used as control of unspecific PKC inhibition. Both CGX1079 and CGX0471 reduced proliferation in response to SEA by 14% and 11%, respectively, compared to untreated control, whereas Sotrastaurin/AEB071 1 μM completely abrogated T-cell proliferation (Fig. 2A). Cell viability was similar between treated and untreated cells (data not shown).

Proliferation through cell cycle is regulated by cyclins and their catalytic subunits, the cyclin-dependent kinases (CDK). Therefore, we also analyzed the effect of

CGX1079 and CGX0471 on the expression of cyclin A2 and its associate Cdc2/Cdk1, which are important for S-phase progression, G2-M transition [84], and SAMHD1 phosphorylation at T592 [28]. PBMCs were treated with PHA/IL-2 before or after adding CGX1079 or CGX0471 10 μM to the culture medium. Gel bands were quantified by densitometry and represented as a bar diagram with the statistical significance. The expression of both cyclin A2 and Cdc2/Cdk1 was 1.6- and 2.0-fold reduced, respectively, in PBMCs pretreated with the compounds before adding PHA/IL-2 to the culture medium (Fig. 2B). However, CGX1079 and CGX0471 had lower effect on reducing the expression of cyclin A2 and Cdc2/Cdk1 ($p < 0.05$) (1.25- and 1.1-fold, respectively) when PBMCs were activated with PHA/IL-2 for 48 hours before adding the PKCθ inhibitors. Therefore, the efficacy of CGX1079 and CGX0471 improved when PBMCs were first treated before activating with PHA/IL-2. IC_{50} in PBMCs pretreated with CGX1079 or CGX0471 at different concentrations (1 nM - 10 μM) decreased to 3.0 μM for CGX1079 and 7.3 μM for CGX0471 (Fig. 2C). SI improved to 15.17 in cells pretreated with CGX1079 and 5.8 with CGX0471.

3.3. Effect of CGX1079 and CGX0471 on the activity of CD8+ T cells from PBMCs of HIV-1 infected patients

Due to the role of CD8+ T cells to clear HIV-1 infection, the effect of PKCθ inhibition by CGX1079 and CGX0471 was analyzed by ELISPOT to measure the antigen-specific IFN-γ production from CD8+ T cells from PBMCs of chronically treated, asymptomatic HIV-1 individuals (Fig. 2D). CGX0471 and CGX1079 reduced 1.7- ($p<0.05$) and 1.9-fold ($p<0.01$), respectively, the CD8+ T-cell activity, whereas Sotrastaurin/AEB071 1 μM completely eliminated the antigen-specific IFN-γ production.

3.4. CGX1079 and CGX0471 acted after viral entry

In order to determine whether CGX0471 and CGX1079 affected HIV-1 replication at the viral entry, PBMCs activated with PHA/IL-2 for 48 hours were infected with VSV-pseudotyped ΔEnv-NL4.3-Luc, which infects cells independently of receptors CD4 and CCR5 or CXCR4. The same procedure explained above for classical viral assays was followed. Both CGX1079 and CGX0471 were very effective for inhibiting the replication of VSV-pseudotyped ΔEnv-NL4.3-Luc (IC_{50}=677.1nM and 675.4nM, respectively) (Fig. 3A), proving that they were acting subsequently to the viral entry via HIV-specific receptor interaction. This was also determined by the ratio between the IC_{50} obtained with VSV-pseudotyped ΔEnv-NL4.3-Luc virus regarding the NL4-3_Renilla strain (Fig. 3B). The lower the ratio (< 1), the lower was the dependency from the viral entry. Neither CGX1079 nor CGX0471 modified the expression on the cell surface of CD4, CCR5 or CXCR4 in PBMCs treated with these compounds at 10 μM for 48 hours (Fig. 3C).

As HIV-1 modulates the actin cytoskeleton to facilitate entry into host cells [85], we analyzed the effect of PKCθ inhibition by CGX1079 or CGX0471 on F-actin polymerization in response to the chemotactic stimulus SDF-1α. Actin polymerization and depolymerization is necessary for chemotaxis [86] and therefore, this experiment could also provide information about the ability of T cells to polarize and migrate in the presence of the PKCθ inhibitors. CGX1079 and CGX0471 reduced in 12% and 25.6%, respectively, F-actin polymerization in response to SDF-1α (Fig. 3D).

3.5. CGX1079 and CGX0471 interfered with HIV-1 retrotranscription and proviral integration

PBMCs treated with CGX1079 or CGX0471 at 10 μM 30 minutes before adding PHA/IL-2 to the culture medium were infected with NL4-3_Renilla strain by spinoculation. Seven hours post-infection, total DNA was extracted and viral retrotranscription was analyzed by quantitative PCR (qPCR). CGX1079 and CGX0471 reduced 2.0-and 1.9-fold, respectively, early retrotranscription ($p<0.01$), and 2.1- and 1.6-fold, respectively, late retrotranscription ($p<0.05$) (Fig. 4A). Five days post-infection, total DNA was extracted to analyze the accumulation of 2-LTR circles and proviral integration. CGX1079 reduced 5.0-fold the accumulation of 2-LTR circles in the nucleus ($p<0.01$) and 8.9-fold the proviral integration ($p<0.001$), whereas CGX0471 reduced 2.6-fold the accumulation of 2-LTR circles ($p<0.05$) and 5.8-fold the proviral integration ($p<0.001$) (Fig. 4B). Total viral DNA was reduced 2.5-fold by both CGX1079 and CGX0471 ($p<0.001$).

3.6. CGX1079 and CGX0471 interfered with SAMHD1 phosphorylation at residue T592

Both CGX1079 and CGX0471 reduced the ability of CD4+ T cells to proliferate (Fig. 2A) and the expression of essential cyclins and kinases related to cell cycle progression such as cyclin A2 and Cdc2/Cdk1 (Fig. 2B), which have been described as responsible for SAMHD1 phosphorylation at T592 [28]. We analyzed then whether treatment with CGX1079 or CGX0471 could be interfering with SAMHD1 phosphorylation at T592. First, we analyzed by immunoblotting the kinetics of SAMHD1 phosphorylation in CD4+ T cells isolated from PBMCs of healthy donors that were treated with PHA/IL-2 for 5 days. SAMHD1 phosphorylation at T592 occurred in CD4+ T cells 2 days after adding PHA/IL-2 and was sustained after 5 days ($p < 0.05$ for 5 days after treatment) (Fig. 5A), correlating with the expression of Cdc2/Cdk1. Second, PBMCs were treated with serial dilutions of CGX1079 or CGX0471 for 30 minutes before adding PHA/IL-2 to the culture medium and

incubating for 5 days. CGX1079 and CGX0471 were only added at the beginning of the experiment. CGX1079 reduced 5-fold SAMHD1 phosphorylation at 5 and 10 μM, whereas CGX0471 reduced 1.6-fold SAMHD1 phosphorylation at 5 μM and 5-fold at 10 μM ($p < 0.05$ for treatment with 10 μM) (Fig. 5B). CD4+ T cells were isolated from PBMCs to determine if SAMHD1 phosphorylation was regulated by CGX1079 specifically in this major HIV-1 target. CD4+ T cells were left untreated or treated with CGX1079 5 μM 30 minutes before adding IL-2 to the culture medium. IL-2 induced SAMHD1 phosphorylation after 2 days and was highest after 5 days ($p < 0.05$) (Fig. 5C). In cells treated with CGX1079, SAMHD1 phosphorylation was delayed until the fourth day and did not reach the same levels than with IL-2 only ($p < 0.001$). Because PMA/ionomycin activates all PKC isoforms [87], purified CD4+ T cells were treated with both stimuli in the presence or not of CGX1079 (Fig. 5D). PMA/ionomycin induced SAMHD1 phosphorylation after 3 days of treatment, and this phosphorylation was delayed by CGX1079 ($p < 0.01$). CGX1079 was only added at the beginning of the experiment. All gel bands were analyzed by densitometry and results were displayed in bar diagrams showing the statistical significance.

SAMHD1 is active when is not phosphorylated, depleting the intracellular dNTP pool and making the resting CD4+ T cells refractory to HIV-1 infection [24]. As CGX1079 delayed SAMHD1 phosphorylation, we tried to rescue viral replication in PBMCs treated with CGX1079 and activated with PHA/IL-2 by adding dNTPs to the culture medium. These cells were infected with NL4-3_Renilla and the synthesis of Renilla was measured in the cell lysates 48 hours post-infection. CGX1079 10 μM reduced 2.5-fold the synthesis of Renilla. Viral replication increased 4.8-fold when dNTPs were added to the culture medium but it was not completely rescued in the

presence of CGX1079 (p<0.001) (Fig. 5E), suggesting that CGX1079 was acting at other level on HIV-1 replication besides retrotranscription.

3.8. CGX1079 and CGX0471 interfered with PKCθ-dependent transcription factors: NF-κB, NF-AT, and AP-1

TCR-mediated activation of PKCθ initiates a cascade of events that culminates in the activation of transcription factors such as NF-κB, NF-AT, and AP-1 [22] that are essential for HIV-1 replication [21]. We reported previously that rottlerin inhibited NF-κB activity but not NF-AT [56]. However, both CGX1079 and CGX0471 interfered with NF-κB, NF-AT, and AP-1 activity in Jurkat E6-1 cells transfected with luciferase expression vectors under the control of promoters with consensus sequences for NF-κB (pκB-LUC), NF-AT (pNFAT-LUC), or AP-1 (pAP1-LUC) (p<0.001), even at the lowest concentration (1.25 μM) (Fig. 6A). Consequently, CGX1079 and CGX0471 also inhibited LTR-dependent transcription in Jurkat E6-1 transfected with a luciferase expression vector (pLTR-LUC) under the control of the HIV-1 LTR promoter and treated with these inhibitors for 30 minutes before adding PMA and incubate for 18 hours (Fig. 6B). CGX1079 and CGX0471 were the most effective inhibitors for interfering with LTR-dependent transcription.

3.9. CGX1079 and CGX0471 hindered the translocation of phospho-T538 PKCθ to the plasma membrane

Jurkat E6-1 were treated or not with CGX1079 or CGX0471 at 10 μM for 6 hours and then the subcellular localization of phospho-T538 PKCθ was analyzed by immunofluorescence. In unstimulated cells, the phosphorylation and translocation of PKCθ was observed only in dividing cells, as was determined by nuclear staining with

Dapi (Fig. 7A). Intensity per pixel was quantified in every image and results were displayed in bar diagrams. In cells treated with CGX1079 or CGX0471, dividing cells showed PKCθ phosphorylation at T538 but it was not translocated to the plasma membrane, which is essential for most PKCθ-mediated downstream signaling pathways in T cells [22]. The levels of cytosolic phospho-T538 PKCθ showed that CGX1079 or CGX0471 also interfered with the phosphorylation of PKCθ at T538. As PMA is able to promptly induce the translocation of PKCθ phosphorylated at T538 to the plasma membrane [56], protein extracts from Jurkat E6-1 treated or not with PMA for 15 minutes in the presence of CGX1079 or CGX0471 were analyzed by immunoblotting (Fig. 7B). As occurred in spontaneously dividing cells, translocation to the plasma membrane of phospho-T538 PKCθ was inhibited when PMA-activated cells were treated with CGX1079 or CGX0471. A decrease in PKCθ expression ($p < 0.05$) and its phosphorylation at T538 ($p < 0.01$) was also observed.

Similar results were obtained in PBMCs treated with CGX1079 ($p < 0.001$) or CGX0471 ($p < 0.05$) 30 minutes after adding PHA/IL-2 to the culture medium and incubated for 48 hours (Fig. 7C). Intensity per pixel was quantified in every image and results were displayed in bar diagrams.

3.10. HIV-1 infection induced PKCθ phosphorylation at T538 in non-dividing CD4+ T cells

PKCθ activity was then essential for HIV-1 replication and we analyzed whether HIV-1 infection could promote the activation of PKCθ in the absence of stimuli in order to trigger its own replication. Jurkat cells were infected with NL4-3_wt strain and incubated for 7 days. PKCθ phosphorylation at T538 was analyzed by immunofluorescence and infected cells were identified by simultaneous staining with a

specific antibody against HIV-1 Tat protein. Uninfected cells were used to determine the basal level of phospho-T538 PKCθ in the absence of stimulus. Intensity per pixel was quantified in every image and results were displayed in bar diagrams. Phospho-T538 PKCθ increased after 7 days of infection in HIV-1 infected, non-dividing cells ($p < 0.001$) (Fig. 8A). This result was confirmed in Jurkat cells transiently transfected with pNL4-3_wt expression vector or an empty pcDNA3 vector as negative control and incubated for 7 days. Levels of phospho-T538 PKCθ increased 5.2-fold in the plasma membrane of infected cells, in the absence of stimulus ($p < 0.001$) (Fig. 8B).

In order to directly determine whether the over-expression of PKCθ could enhance HIV-1 replication, Jurkat E6-1 cells were co-transfected with an infectious NL4-3_Renilla clone and an expression vector of wild-type PKCθ (pPKCθ-wt), or a constitutively active PKCθ mutant (pPKCθ-A/E), or a dominant negative PKCθ mutant (kinase inactive) (pPKCθ-K/R) [63]. Transfection of an empty pcDNA3 vector was used as negative control. Overexpression of PKCθ-wt increased 1.5-fold HIV-1 replication in the absence of activation, regarding viral replication in basal conditions (pcDNA3) (Fig. 8C). Overexpression of the constitutively active mutant PKCθ-A/E increased 2.5-fold HIV-1 replication ($p<0.01$), whereas co-transfection with inactive PKCθ-K/R expression vector caused 2-fold decreased in HIV-1 replication.

4. Discussion

ART changed HIV-1 infection into a chronic disease that requires long-life treatment. Although morbidity related to the infection has significantly decreased, the adverse effects related to ART constitute an additional motivation to completely eradicate the infection. To fulfill this goal, several pharmacological compounds have been tested to reactivate and destroy the latent reservoirs [88], PKC agonists such as bryostatin-1 being the most promising choice [89]. Unfortunately, this strategy of "shock and kill" has proved to be unsuccessful up till now as further measures are needed in order to completely deplete the reservoirs [89]. Although these strategies are most needed to purge the reservoirs from patients with chronic infection, new strategies should also be developed to impede the formation of latent reservoirs. Early ART can decrease the size of the reservoir [14-16], which has been related to a better prognosis and a long-term control of viraemia in patients under treatment interruption [12,13]. At present, a total inhibition of the reservoir seeding seems impossible as it begins very early after the infection [5,36,90]. As the generation of the reservoir seems to begin before the clinical onset of the infection [5], likely an early treatment would not be early enough to fully prevent the reservoir seeding [91]. But what seems clear is that the sooner the ART begins, the lower the size of the reservoir and the longer the time until viral rebound [5]. Therefore, new strategies should be developed in order to decrease the size of the reservoir as much as possible.

Although several cell types might be contributing to the viral reservoir, the memory resting CD4+ T cells are likely the main target of the virus [92-95]. Reservoir seeding begins during acute HIV-1 infection, when viraemia reaches high levels [1] due to the massive activation of CD4+ T cells [6,7]. PKCθ is critical to regulate mature T-cell activation, proliferation, and differentiation [96-98], and it shows a quite restricted

pattern of expression, with high levels in T cells [74,99]. Therefore, the selective inhibition of PKCθ would not cause a general immunosuppression but it will affect specifically the main target of HIV-1 infection: the CD4+ T cells. The possibility that CD8+ T cells were also affected was a concern as they are essential for the control of viraemia during primary infection [100] and this risk should be avoided.

In previous work, we showed the essential role of PKCθ in HIV-1 infection [56]. Here we demonstrated that there was a feedback cycle where not only the increased PKCθ activity enhanced HIV-1 replication, but the own viral infection also induced a high phosphorylation of PKCθ in its activation loop. Therefore, the possibility of using PKCθ as a target against HIV-1 should be considered. For this purpose, we analyzed the ability to control HIV-1 replication of seven compounds developed by different laboratories. Two PKCθ specific inhibitors -CGX1079 and CGX0471- showed the best potency and safety in vitro and were selected for further analysis. CGX1079 and CGX0471 rendered PBMCs less susceptible to HIV-1 infection, as both compounds reduced PKCθ-dependent T-cell activation even in the presence of classical stimuli such as PHA and IL-2. PHA stimulates T cells by cross-linking the TCR/CD3 complex [101], which will in turn initiate PKCθ signaling pathways. CGX1079 and CGX0471 reduced PKCθ levels and interfered with PKCθ phosphorylation at T583 and its translocation to the plasma membrane. Both last events are necessary to trigger downstream signaling pathways that end in the activation of NF-κB, NF-AT, and AP-1 [22], which are essential for HIV-1 transcription [21]. Therefore, one of the major inhibitory effects of CGX1079 and CGX0471 on HIV-1 replication was at the transcriptional level. But these compounds also inhibited SAMHD1 phosphorylation at T592 mediated by PHA/IL-2 or PMA/ionomycin. SAMHD1 protects resting CD4+ T cells from HIV-1 infection as long as it is not phosphorylated at T592, as its effects on

dNTP pool depletion [28] or RNase activity on the viral RNA [27] are then lost. Treatment of PBMCs with PHA/IL-2 induced SAMHD1 phosphorylation, allowing viral infection, but previous treatment with CGX1079 or CGX0471 interfered with this phosphorylation, impeding HIV-1 full retrotranscription. However, the main mechanism of PKCθ inhibitors relied on the interference with the viral transcription as the addition of dNTPs to the culture medium to counteract the depletion induced by SAMHD1 did not completely restore viral replication.

One intriguing result was the different concentration of PKCθ inhibitor required to inhibit HIV-1 replication in PBMCs that were activated with PHA/IL-2 before or after treatment with CGX1079 or CGX0471. A possible explanation might be that in resting PBMCs the absence of activation would promote PKCθ degradation by the ubiquitin-protein ligase Cbl-b [49]. In this scenario, when resting PBMCs were treated with PKCθ inhibitors, Cbl-b-mediated PKCθ degradation would be enhanced and less quantity of PKCθ inhibitor would be needed to interfere with HIV-1 replication. As a consequence, T-cell activation would be more difficult and the level of SAMHD1 phosphorylation would be low. On the contrary, on PBMCs treated with PHA/IL-2, PKCθ is activated and initiates downstream signaling pathways, cell cycle progression, and full SAMHD1 phosphorylation. Cbl-b is then degraded by PKCθ [55] and higher concentration of PKCθ inhibitors would be required to control the kinase activity.

The effect of CGX1079 and CGX0471 on the viral entry was ruled out. They did not cause the internalization of receptors such as CD4, CCR5, or CXCR4 and, surprisingly, a higher effect of CGX1079 and CGX0471 on HIV-1 replication was observed when the viral entry occurred through the VSV envelope. This could be explained because the functional pathway of VSV entry is via clathrin-coated pits [102] and PKCθ is involved in cytoskeleton reorganization and vesicle trafficking [103].

Likely, PKCθ inhibitors were interfering with the traffic of clathrin-coated vesicles, specially affecting the VSV entry.

Finally, although both CGX1079 and CGX0471 caused some delay in T-cell proliferation and migration, they did not caused anergy, supporting the fact that the inhibition of PKCθ would control HIV-1 replication specifically at its major target, the CD4+ T cells, without affecting their functionality. A general immunosuppressive effect would be avoided as PKCθ has been described as dispensable for immune responses against several viruses or cancer. In fact, PKCθ knockdown does not impair Th1 responses or CD8-mediated protection from viral infection [104,105], likely due to compensatory activities mediated by innate immunity mechanisms [106-108]. In accordance, although both CGX1079 and CGX0471 partly interfered with the release of IFNγ from CD8+ T cells of HIV-infected patients, this interference was not absolute as it also occurred with the pan-PKC inhibitor AEB071/Sotrastaurin.

In conclusion, we described the mechanism of action of specific PKCθ inhibitors for the control of HIV-1 replication. Early treatment with ART and simultaneous restraint of CD4+ T cell activation by using selective PKCθ inhibitors as adjuvant would reduce the size of the reservoir, improve CD4 count, and preserve the immune response integrity. In fact, PKCθ suppression can be considered as a physiologic event as it occurs normally in regulatory T cells due to PKCθ sequestration away from the immunological synapse [109,110]. Further studies in vivo are required in order to determine the feasibility of this approach.

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Figure legends

Fig. 1. Analysis of IC_{50} and CC_{50} of seven PKCθ inhibitors for the control of HIV-1 infection. (A) PBMCs from healthy donors and activated for 2 days with PHA/IL-2 were infected with NL4-3_Renilla clone in 96-well microtiter plates in the presence of increasing concentrations of each inhibitor. Production of Renilla (RLUs) was measured 48h post-infection. All measurements were done in triplicate and mean is represented with the standard error of the mean (SEM). R^2 is a measure of goodness-of-fit of linear regression. (B) Analysis by bright-field microscopy of the formation of syncytia in MT-2 cells infected with NL4-3_wt for 5 days in the presence or absence (untreated) of PKCθ inhibitors. White arrows indicate the position of the syncytia. (C) MT-2 cells treated with different PKCθ inhibitors (Rottlerin 3 μM; PF-05263557 12.5 μM; CGX1079 10 μM; CGX0471 10 μM), or left untreated, were infected for 7 days with NL4-3_Renilla by spinoculation. Production of Renilla (RLUs) was measured after 1, 3, and 7 days of infection. All measurements were done in triplicate and mean is represented with SEM.

Fig. 2. Analysis of the effect of CGX1079 and CGX0471 on T cells proliferation and CD8-medianted release of IFNγ. (A) Analysis by CFSE staining of the proliferation of PBMCs in response to SEA and in the presence of PKCθ inhibitors or the pan-PKC inhibitor Sotrastaurin. The number of events per generation is indicated (Gn), as well as the percentage of proliferating cells regarding to cells in resting conditions (without SEA). (B) Immunoblotting of protein extract obtained from CD4+ T cells isolated from PBMCs of healthy donors and treated with CGX1079 or CGX0471 before adding PHA/IL-2 or 2 days after treatment with PHA/IL-2. Specific antibodies against Cyclin A2 and Cdc2/Cdk1 were used. β-actin was used as internal loading control. Gel bands were quantified by densitometry and values were represented in bar diagrams showing

the statistical significance. (C) PBMCs from healthy donors treated with CGX1079 or CGX0471 before adding PHA/IL-2 and incubated for 48 hours. Cells were then infected with NL4-3_Renilla clone in 96-well microtiter plates in the presence of increasing concentrations of each inhibitor. Production of Renilla (RLUs) was measured 48h post-infection. All measurements were done in triplicate and mean is represented with SEM. (D) Measurement by ELISPOT of antigen-specific IFN-γ production from CD8+ T cells from HIV-infected patients that were treated with PKCθ inhibitors or the pan-PKC inhibitor Sotrastaurin for 48 hours. Different HLA class I-restricted synthetic peptides from Gag, Pol, Env and Nef proteins and peptide pools spanning the entire HIV-1 consensus B Gag proteins sequences were used (labelled as HIV), as well as a positive control formed by a pool of MHC class I-restricted T cell epitopes from human cytomegalovirus, Epstein-Barr virus and influenza virus (labelled as CEF). PHA was used as additional positive control. RPMI/10%FCS was used as negative control (labelled as RPMI). All measurements were done in triplicate and mean is represented with SEM.

Fig. 3. The inhibition of PKCθ appears to affect HIV-1 replication after viral entry. (A) PBMCs were treated with CGX1079 or CGX0471 before adding PHA/IL-2 and then incubated for 48 hours before infecting with VSV-pseudotyped ΔEnv-NL4-3_Luc clone in 96-well microtiter plates in the presence of increasing concentrations of each inhibitor. Production of Renilla (IC_{50}) and cytotoxicity (CC_{50}) (RLUs) were measured 48h post-infection. All measurements were done in triplicate and mean is represented with SEM. (B) Ratio IC_{50} of CGX1079 and CGX0471 between the infection with VSV-pseudotyped ΔEnv-NL4-3_Luc and NL4-3_Renilla clones. (C) Analysis by flow cytometry of the expression of receptors CD4, CCR5 and CXCR4 in PBMCs after treatment with PKCθ inhibitors, regarding control cells. (D) Analysis by flow cytometry

after staining with phalloidin-FITC of PBMCs migration in response to SDF1α in the presence of PKCθ inhibitors.

Fig. 4. The inhibition of PKCθ blocked HIV-1 replication at retrotranscription and proviral integration. Analysis by qPCR of early and late retrotranscription (A) and proviral integration, formation of 2-LTR circles and total viral DNA (B) in PBMCs treated with CGX1079 or CGX0471 before activation with PHA/IL-2 and infection with NL4-3_Renilla clone, regarding to untreated cells. All measurements were done in triplicate and mean is represented with SEM.

Fig. 5. The inhibition of PKCθ interfered with SAMHD1 phosphorylation at T592. (A) Analysis by immunoblotting of the kinetics of SAMHD1 phosphorylation in CD4+ T cells after treatment with PHA/IL-2. Specific antibodies against total SAMHD1 and phosphorylated at T592 and Cdc2/Cdk1 were used. (B) Analysis of SAMHD1 phosphorylation in PBMCs treated with different concentrations of CGX1079 or CGX0471 before activating for 5 days with PHA/IL-2. (C) Kinetics of SAMHD1 phosphorylation in CD4+ T cells treated or not with CGX1079 10 μM for 5 days before adding IL-2. (D) Kinetics of SAMHD1 phosphorylation in CD4+ T cells treated simultaneously with CGX1079 and PMA/ionomycin for 4 days. In all cases, total SAMHD1 was used as loading control. Gel bands were quantified by densitometry and values were represented in bar diagrams showing the statistical significance. (E) Analysis of the production of Renilla (RLUs) in PBMCs treated with CGX1079 at different concentrations before activation with PHA/IL-2 and infection with NL4-3_Renilla for 48 hours, in the presence or absence of dNTPs 25 μM.

Fig. 6. The inhibition of PKCθ blocked HIV-1 transcription. (A) Analysis of the synthesis of luciferase (RLUs) in Jurkat E6-1 transfected with vectors pAP1-LUC, pκB-LUC or pNFAT-LUC in the presence of different concentrations of CGX1079 or

CGX0471 (1.25 to 10 μM) for 48 hours. (B) Analysis of the synthesis of luciferase (RLUs) in Jurkat E6-1 transfected with a luciferase expression vector under the control of HIV-1 LTR promoter (pLTR-LUC) and treated with BIX02671 (8 μM), PF-05263557 (12.5 μM), CGX1039, CGX1079, CGX0471 (10 μM), or AEB071/Sotrastaurin (1 μM) before adding PMA or not (untreated).

Fig. 7. CGX1079 and CGX0471 decreased PKCθ levels and interfered with phosphorylation at T538 and translocation to the plasma membrane. (A) Analysis by immunofluorescence of PKCθ phosphorylation at T538 in Jurkat E6-1 treated with CGX1079 or CGX0471 10 μM for 48 hours. Specific antibody against PKCθ phosphorylated at T538 and a secondary conjugated to Alexa 546 was used. Lipid rafts were stained with cholera subunit B-FITC and nuclei were stained with Dapi. Intensity mean per pixel was calculated and values were represented in bar diagrams showing statistical significance. (B) Analysis by immunoblotting of protein extracts from plasma membrane and cytosol of Jurkat E6-1 treated with CGX1079 or CGX0471 10 μM for 48 hours. β-actin was used as internal loading control. Gel bands were quantified by densitometry and values were represented in bar diagrams showing statistical significance. (C) Analysis by immunofluorescence of PKCθ phosphorylation at T538 in PBMCs treated with CGX1079 or CGX0471 10 μM before adding PHA/IL-2 for 48 hours. Specific antibody against PKCθ phosphorylated at T538 and a secondary conjugated to Alexa 546 was used. Nuclei were stained with Dapi. Intensity mean per pixel was calculated and values were represented in bar diagrams showing statistical significance.

Fig. 8. HIV-1 infection induced PKCθ phosphorylation at T538. (A) Analysis by immunofluorescence of Jurkat E6-1 cells infected with NL4-3_wt strain for 7 days, in comparison with uninfected cells. Cells were stained with specific antibodies directed

against PKCθ phosphorylated at T538 and HIV-1 protein Tat, and secondary antibodies conjugated with Alexa 546 and Alexa 488, respectively. Nuclei were stained with Dapi. Intensity mean per pixel was calculated and values were represented in bar diagrams showing statistical significance. (B) Analysis by immunoblotting of protein extracts from Jurkat E6-1 cells transfected with pNL4-3_wt infectious clone or pcDNA3 as basal control, and incubated for 7 days. Specific antibodies against PKCθ phosphorylated at T538 and β-actin, as internal loading control, were used. Gel bands were quantified by densitometry and values were represented in bar diagrams showing statistical significance. (C) Analysis of the production of Renilla (RLUs) in the lysates of Jurkat E6-1 cells co-transfected with pNL4-3_Renilla infectious clone and pPKCθ-wt or pPKCθ-A/E or pPKCθ-K/R expression vectors. pcDNA3 empty vector was used as negative control.