

# The Lupane-type Triterpene 30-Oxo-calenduladiol Is a CCR5 Antagonist with Anti-HIV-1 and Anti-chemotactic Activities<sup>\*[5]</sup>

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The existence of drug-resistant human immunodeficiency virus (HIV) viruses in patients receiving antiretroviral treatment urgently requires the characterization and development of new antiretroviral drugs designed to inhibit resistant viruses and to complement the existing antiretroviral strategies against AIDS. We assayed several natural or semi-synthetic lupane-type pentacyclic triterpenes in their ability to inhibit HIV-1 infection in permissive cells. We observed that the 30-oxo-calenduladiol triterpene, compound 1, specifically impaired R5-tropic HIV-1 envelope-mediated viral infection and cell fusion in permissive cells, without affecting X4-tropic virus. This lupane derivative competed for the binding of a specific anti-CCR5 monoclonal antibody or the natural CCL5 chemokine to the CCR5 viral coreceptor with high affinity. 30-Oxo-calenduladiol seems not to interact with the CD4 antigen, the main HIV receptor, or the CXCR4 viral coreceptor. Our results suggest that compound 1 is a specific CCR5 antagonist, because it binds to the CCR5 receptor without triggering cell signaling or receptor internalization, and inhibits RANTES (regulated on activation normal T cell expressed and secreted)-mediated CCR5 internalization, intracellular calcium mobilization, and cell chemotaxis. Furthermore, compound 1 appeared not to interact with  $\beta$ -chemokine receptors CCR1, CCR2b, CCR3, or CCR4. Thereby, the 30-oxo-calenduladiol-associated anti-HIV-1 activity against R5-tropic virus appears to rely on the selective occupancy of the CCR5

receptor to inhibit CCR5-mediated HIV-1 infection. Therefore, it is plausible that the chemical structure of 30-oxo-calenduladiol or other related dihydroxylated lupane-type triterpenes could represent a good model to develop more potent anti-HIV-1 molecules to inhibit viral infection by interfering with early fusion and entry steps in the HIV life cycle.

The human immunodeficiency virus (HIV)<sup>7</sup> pandemic is a medical challenge and represents the public health crisis of our time (1–5). Antiretroviral treatment achieves long-lasting viral suppression and, subsequently, reduces the morbidity and mortality of HIV-infected individuals. However, current drugs do not eradicate HIV infection and lifelong treatment might be needed (2).

Emerging drug-resistant HIV viruses, in patients receiving high active antiretroviral treatment, urgently needs the development of new antiretroviral molecules designed to inhibit resistant viruses, because many patients treated during the past decades harbor viral strains with reduced susceptibilities to many if not all available drugs (2, 6). In this matter, pentacyclic triterpenes represent a varied class of natural products presenting antitumor and antiviral activities (7–9). A well studied pentacyclic lupane-type triterpene is the betulinic acid ( $\beta$ -hydroxy-lup-20(29)-en-28-oic acid), widely distributed throughout the plant kingdom, which presents anti-inflammatory, anti-malarial, and anti-HIV-1 effects *in vitro* (7, 9, 10). Although its mechanism of action has not been fully determined, it has been reported that some lupane-type triterpene derivatives impair HIV-1 fusion through interacting with the viral glycoprotein gp41, or disrupting the assembly and budding of emerging viral particles in infected target cells (reviewed in Ref. 9).

In the present work, we aimed to test the ability of several non-acid lupane-type triterpene, natural or derivative compounds, to inhibit HIV-1 viral infection and to determine the mechanism of action. Our results indicate that the semi-synthetic 30-oxo-calenduladiol, compound 1, specifically interacts

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<sup>7</sup> The abbreviations used are: HIV-1, human immunodeficiency virus type 1; VSV-G, vesicular stomatitis virus G protein; RANTES, regulated on activation normal T expressed and secreted; TIRFM, total internal reflection fluorescence microscopy; PE, phycoerythrin; MCP-1, monocyte chemotactic protein 1; TARC, thymus- and activation-regulated chemokine; EGFP, enhanced green fluorescent protein; EF, evanescent field; IR, infrared; mAb, monoclonal antibody; PBS, phosphate-buffered saline; Env, envelope.

with the G protein-coupled CCR5 chemokine receptor, acting as an antagonist, inhibiting R5-tropic HIV-1 viral infection and CCL5 (regulated on activation normal T expressed and secreted (RANTES) chemokine)-mediated CCR5 internalization, cell signaling, and chemotaxis.

## EXPERIMENTAL PROCEDURES

### Chemistry

**General**—All solvents and reagents were purified by Standard techniques, as previously described (11). All reactions were monitored by thin layer chromatography (TLC) (on silica gel POLYGRAM® SIL G/UV<sub>254</sub> foils). Pre-coated SIL G-100 UV<sub>254</sub> (Machery-Nagel, Düren, Germany) TLC plates were used for preparative TLC purification. <sup>1</sup>H nuclear magnetic resonance spectra were recorded in CDCl<sub>3</sub> or C<sub>6</sub>D<sub>6</sub> at 300 and 400 MHz, using Bruker AMX300 and AMX400 instruments. For <sup>1</sup>H spectra, chemical shifts are given in parts per million (ppm) and are referenced to the residual solvent peak. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad.

Proton assignments and stereochemistry were supported by <sup>1</sup>H-<sup>1</sup>H COSY and ROESY where necessary. Data are reported in the following manner with chemical shift (integration, multiplicity, and coupling constant, if appropriate). Coupling constants (J) are given in Hertz (Hz) to the nearest 0.5 Hz. <sup>13</sup>C NMR spectra were recorded at 75 and 100 MHz using Bruker AMX300 and AMX400 instruments. Carbon spectra assignments were supported by DEPT-135 spectra, <sup>13</sup>C-<sup>1</sup>H (HMQC), and <sup>13</sup>C-<sup>1</sup>H (HMBC) correlations where necessary. Chemical shifts are quoted in ppm and are referenced to the appropriate residual solvent peak. MS and HRMS were recorded at VG Micromass ZAB-2F. IR spectra were taken on a Bruker IFS28/55 spectrophotometer.

**Molecules Assayed**—Six natural or derivative lupanes were assayed (1–6). Compounds 1–3 and 5 were semi-synthesized as described below, whereas natural compounds 4 and 6 were isolated from *Maytenus apurimacensis*, using a previously described method (12–16). The degree of purity of the tested compounds was estimated higher than 99% by NMR spectroscopy.

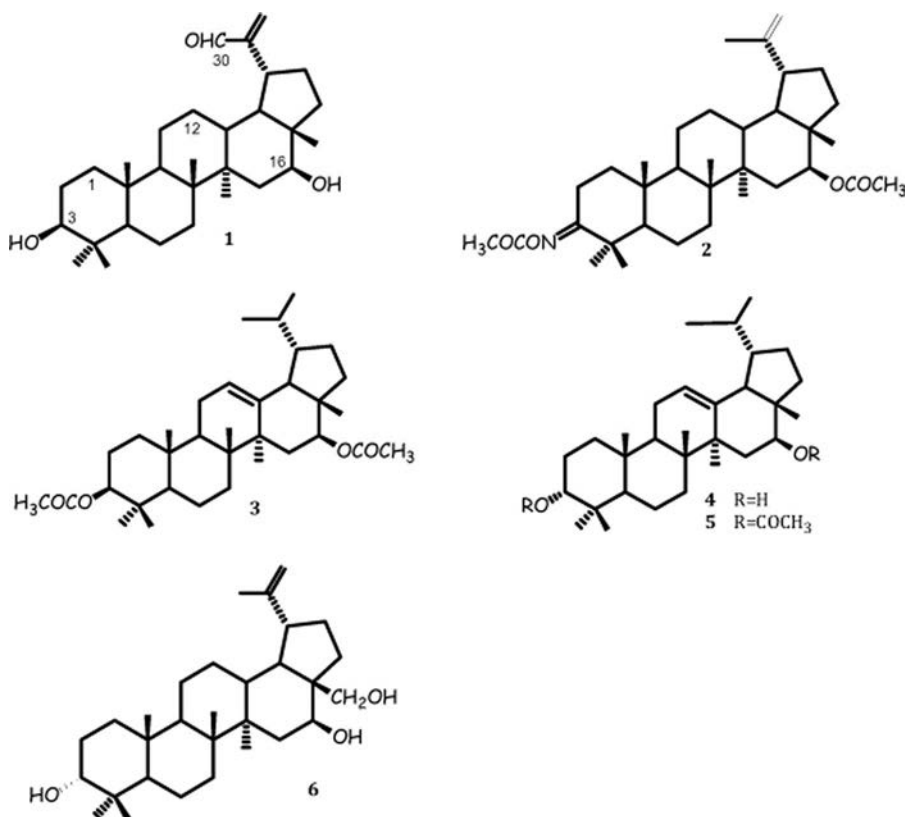
**Preparation of 30-Oxo-calenduladiol (1)**—10 mg (0.023 mmol) of calenduladiol in 2 ml of EtOH were treated with 2.6 mg (1 eq) of SeO<sub>2</sub>. The reaction mixture was heated under reflux for 10 h. Then, the reaction mixture was cooled and EtOH was removed under reduced pressure. The crude was treated with water and extracted three times with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was dried and concentrated under reduced pressure. The residue was purified by preparative TLC with hexanes/EtOAc (7:3) as solvent to obtain 3.6 mg (30.4%) of 1 as amorphous pale yellow solid. Compound 1 showed identical spectroscopic data to those previously reported (16).

**Preparation of Resinone Oxime Diacetate (2)**—15 mg (0.035 mmol) of resinone (17) in 2 ml of EtOH were treated with 7.3 mg (3 eq) of hydroxylamine hydrochloride and a solution of 5.75 mg (2 eq) of sodium acetate in 0.5 ml of H<sub>2</sub>O. The reaction mixture was heated under reflux for 15 h. Then, the reaction mixture was cooled and the solvent was removed under reduced pressure. The crude was treated with water and

extracted three times with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was dried and concentrated under reduced pressure. The residue was purified by preparative TLC with hexanes/EtOAc (7:3) as solvent to obtain 13.0 mg (80%) of resinone oxime as amorphous pale yellow solid. Then 6 mg (0.013) of resinone oxime in 2 ml of CH<sub>2</sub>Cl<sub>2</sub> were treated with pyridine, the catalytic amount of 4-dimethylaminopyridine, and an excess of Ac<sub>2</sub>O (10 μl). The reaction mixture was stirred at room temperature for 10 h. Then the solvent was removed under reduced pressure and the residue was purified by TLC preparative with hexanes/EtOAc (4:1) as solvent to afford 2.6 mg (38.5%) of compound 2 as an amorphous solid: [α]<sub>D</sub><sup>20</sup>, +4.6 (c 0.26, CHCl<sub>3</sub>); UV (EtOH) λ<sub>max</sub> (log ε) nm, 340 (2.42); 273 (2.72); IR (CHCl<sub>3</sub>) ν<sub>max</sub>, 2925, 2854, 1735, 1459, 1370, 1023, 755 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ, 4.89 (1H, dd, J = 8.5; 3.9 Hz, H-16), 4.72 (1H, s, H-29b), 4.62 (1H, s, H-29a), 2.20 (3H, s, OCOCH<sub>3</sub>), 2.04 (3H, s, OCOCH<sub>3</sub>), 1.69 (3H, s, H-30), 1.14 (3H, s, H-26), 1.08 (H, s, H-23), 1.05 (3H, s, H-27), 0.94 (3H, s, H-24), 0.87 (6H, s, H-25, H-28); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ, 170.0 (2xs, OCOCH<sub>3</sub>), 167.0 (s, C-3), 149.5 (s, C-20), 109.7 (t, C-29), 78.7 (d, C-16), 54.9 (d, C-5), 49.3 (d, C-9), 47.3 (d, C-18), 47.1 (d, C-19), 47.0 (s, C-17), 43.9 (s, C-14), 40.9 (s, C-4), 40.7 (s, C-8), 38.9 (t, C-1), 37.4 (t, C-22), 37.3 (d, C-13), 36.9 (s, C-10), 33.6 (t, C-15), 33.3 (t, C-7), 31.6 (t, C-2), 29.1 (t, C-21), 27.2 (c, C-23), 24.3 (t, C-12), 22.4 (2xc, C-24, C-11), 21.1 (2xc, OCOCH<sub>3</sub>), 20.8 (t, C-6), 19.8 (c, C-30), 15.8 (c, C-25), 15.6 (c, C-26), 13.8 (c, C-27), and 12.4 (c, C-28); EIMS m/z (%), 481 [M<sup>+</sup>-C<sub>2</sub>H<sub>5</sub>ON] (40); 466 (6); 452 (1); 422 (5); HREIMS m/z (%), 481.3688 (calculated for C<sub>32</sub>H<sub>49</sub>O<sub>3</sub> 481.3682), 452.3545 (M<sup>+</sup>-C<sub>4</sub>H<sub>8</sub>O<sub>2</sub>) (calculated for C<sub>30</sub>H<sub>46</sub>O<sub>2</sub>N, 452.3529).

**Preparation of Compound 3 (3β,16β-Diacetyl-lup-12-ene)**—5.8 mg (0.01 mmol) of 3β,16β-dihydroxylup-12-ene were acetylated with 2.5 eq of Ac<sub>2</sub>O (2.5 μl), following the same procedure described for compound 2. The residue was purified by TLC preparative with hexane/EtOAc (4:1) as solvent to yield 6.8 mg (86%) of compound 3 as an amorphous white solid: [α]<sub>D</sub><sup>20</sup>, +36.4 (c, 0.6, CHCl<sub>3</sub>); UV (EtOH) λ<sub>max</sub> (log ε) nm, 258 (3.09); IR (CHCl<sub>3</sub>) ν<sub>max</sub>, 2926, 2855, 1737, 1456, 1368, 1243, and 1025 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ, 5.45 (1H, dd, J = 11.3; 5.4 Hz, H-16), 5.19 (1H, t, J = 3.4 Hz, H-12), 4.50 (1H, dd, J = 5.5; 9.0 Hz, H-3), 2.04 (3H, s, OCOCH<sub>3</sub>), 2.02 (3H, s, OCOCH<sub>3</sub>), 1.18 (3H, s, H-27), 1.02 (3H, s, H-26), 0.97 (3H, s, H-23), 0.92 (3H, s, H-25), 0.87 (3H, s, H-24), 0.86 (3H, d, J = 7.7 Hz, H-29), 0.85 (3H, s, H-28), 0.79 (3H, d, J = 6.2 Hz, H-30); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ: 170.7 (s, OCOCH<sub>3</sub>), 170.5 (s, OCOCH<sub>3</sub>), 137.3 (s, C-13), 124.9 (d, C-12), 80.6 (d, C-3), 70.5 (d, C-16), 60.5 (d, C-18), 54.9 (d, C-5), 46.5 (d, C-9), 43.6 (s, C-14), 39.8 (s, C-8), 39.2 (2xd, C-19, C-20), 38.1 (t, C-1), 37.4 (s, C-17), 37.3 (s, C-4), 36.4 (s, C-10), 35.1 (t, C-15), 32.5 (t, C-7), 32.0 (t, C-22), 30.3 (t, C-21), 27.8 (c, C-23), 24.8 (c, C-27), 23.3 (t, C-2), 23.1 (t, C-11), 22.4 (c, C-28), 21.0 (C-30), 20.9 (2xc, OCOCH<sub>3</sub>), 17.9 (t, C-6), 17.3 (c, C-25), 16.6 (c, C-26), 16.4 (t, C-29), 15.5 (c, C-24); EIMS m/z (%), 526 [M<sup>+</sup>] (1), 466 (16), 451 (7); HREIMS, 526.4029 (calculated for C<sub>34</sub>H<sub>54</sub>O<sub>4</sub>, 526.4022), 466.3778 (M<sup>+</sup>-C<sub>2</sub>H<sub>4</sub>O<sub>2</sub>) (calculated for C<sub>32</sub>H<sub>50</sub>O<sub>2</sub>, 466.3811).

**Preparation of Compound 5 (3α,16β-Diacetyl-lup-12-ene)**—5.9 mg (84.6%) of compound 5 as amorphous solid were obtained from 6.0 mg (0.013 mmol) of 3α,16β-dihydroxylup-12-ene under identical reaction and purification conditions as



#### Lupane-type triterpene molecules assayed

FIGURE 1. Chemical structures of a series of lupane-type triterpenes assayed in their capacity to inhibit HIV-1 infection. Compounds 1-3 and 5 are semi-synthetic lupane triterpenes, whereas compounds 4 and 6 are natural triterpenes isolated from *M. apurimacensis*.

those used for compound 3:  $[\alpha]_D^{20}$ , +11.2 (c 0.6,  $\text{CHCl}_3$ ); UV (EtOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ), 202 (3.79) nm; IR ( $\text{CHCl}_3$ )  $\nu_{\text{max}}$ , 2926, 2856, 1737, 1457, 1372, 1244, 1024, and 756  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ , 5.47 (1H, dd,  $J = 11.4$ ; 5.4 Hz, H-16), 5.20 (1H, t,  $J = 3.4$ , H-12), 4.63 (1H, bs, H-3), 2.08 (3H, s,  $\text{OCOCH}_3$ ), 2.03 (3H, s,  $\text{OCOCH}_3$ ), 1.24 (3H, s, H-27), 1.22 (3H, d,  $J = 7.1$  Hz, H-29), 1.03 (3H, s, H-26), 0.97 (3H, s, H-25), 0.89 (3H, s, H-23), 0.87 (6H, s, H-28, H-24), and 0.80 (3H, d,  $J = 6.3$  Hz, H-30);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$ , 170.6 (2xs,  $\text{OCOCH}_3$ ), 137.2 (s, C-13), 125.1 (d, C-12), 77.8 (d, C-3), 70.5 (d, C-16), 60.5 (d, C-18), 49.7 (d, C-5), 46.4 (d, C-9), 43.6 (s, C-14), 40.0 (s, C-8), 39.2 (2xd, C-19, C-20), 37.3 (s, C-17), 36.5 (s, C-4), 36.2 (s, C-10), 35.1 (t, C-7), 33.6 (t, C-1), 32.4 (t, C-15), 32.1 (t, C-22), 30.3 (t, C-21), 27.5 (c, C-23), 24.1 (c, C-27), 23.0 (t, C-2), 22.9 (c, C-24), 22.4 (t, C-11), 21.7 (c, C-28), 21.1 (c,  $\text{OCOCH}_3$ ), 21.0 (c,  $\text{OCOCH}_3$ ), 20.9 (c, C-29), 17.8 (t, C-6), 17.3 (c, C-30), 16.6 (c, C-26), and 15.2 (t, C-25); EIMS  $m/z$  (%), 526 [ $\text{M}^+$ ] (1), 466 (14), 451 (6); HREIMS: 526.4038 (calculated for  $\text{C}_{34}\text{H}_{54}\text{O}_4$ , 526.4022), 467.3917 ( $\text{M}^+ - \text{OCOCH}_3$ ) (calculated for  $\text{C}_{32}\text{H}_{51}\text{O}_2$ , 467.3889).

#### Antibodies and Reagents

The monoclonal antibodies (mAbs) CD184 (clone 12G5) and CD195 (clone 2D7/CCR5), used as phycoerythrin (PE) conjugates (BD Bioscience/BD Pharmingen, San Jose, CA), are directed against the second extracellular loop of CXCR4 and CCR5, respectively. The PE-labeled mAb RPT-4 is a neutral-

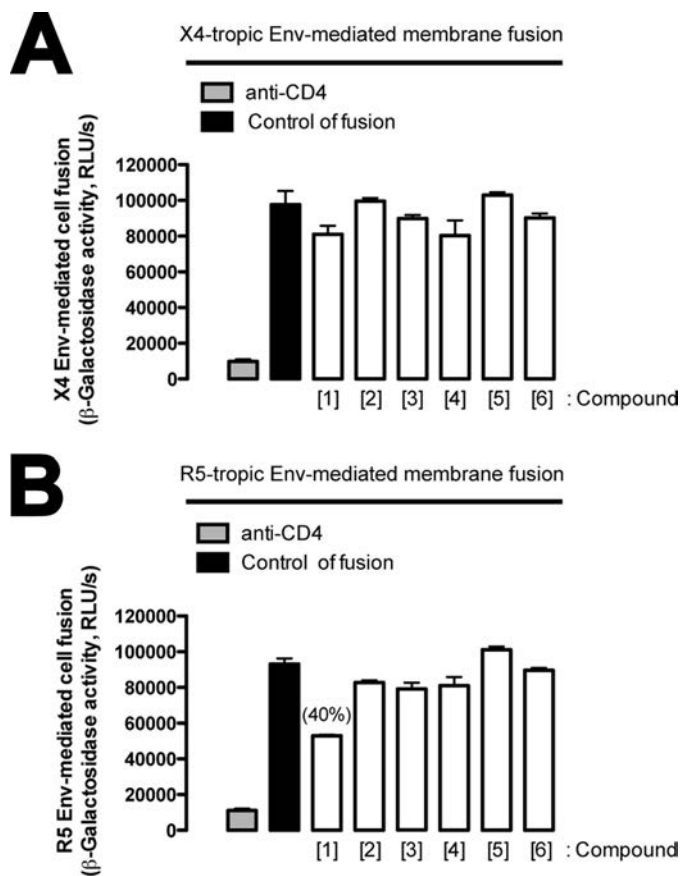
izing antibody against CD4 (eBioscience, San Diego, CA). PE-conjugated mAbs against human CCR1 (FAB145P), CCR2b (FAB151P), CCR3 (FAB155P), and CCR4 (FAB1567P) chemokine receptors were from R&D Systems (Minneapolis, MN). The Cremophor<sup>®</sup> EL emulsifying agent that is used in aqueous preparations of hydrophobic substances was from Sigma. In all experiments, 30-oxo-calenduladiol and the other assayed triterpenes were dissolved in the following working buffer solution: Cremophor EL/dimethyl sulfoxide/culture medium at a ratio of 1:1:8 (v/v/v). The Fura 2-AM probe was from Invitrogen. The Fluorokine<sup>™</sup> human biotinylated RANTES (CCL5), monocyte chemotactic protein 1 (MCP-1), Eotaxin, or thymus- and activation-regulated chemokine (TARC) kits, and the human recombinant RANTES were from R&D Systems.

#### Cells

The human CEM.NKR-CCR5 permissive cell line (catalog number 4376, NIH AIDS Research and Reference Reagent Program) was grown at 37 °C in a humidified atmosphere with 5%  $\text{CO}_2$  in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (Lonza), 1% L-glutamine, and 1% penicillin-streptomycin antibiotics. Cells were regularly passaged every 3 days. The 293T cell line was similarly cultured, in supplemented Dulbecco's modified Eagle's medium (Lonza), and were regularly passaged every 2-3 days. 24 h before cell transfection with viral or human DNA constructs, cells were harvested and resuspended at a density of 50-70% in fresh supplemented Dulbecco's modified Eagle's medium. The HeLa-P5 cells, stably transfected with human CD4 and C-terminal enhanced green fluorescent protein (EGFP)-tagged CCR5 cDNAs and with an HIV-long terminal repeat-driven  $\beta$ -galactosidase reporter gene (18), as well as, HeLa-243 and HeLa-ADA cells, co-expressing the Tat and X4- and R5-tropic HIV-1-Env proteins, respectively, were provided by Dr. M. Alizon (Hôpital Cochin, Paris, France) (18, 19). Human astroglia U87 cell line, stably expressing human CD4 and CCR3 receptors (U87.CD4.CCR3), was kindly provided by Dr. Guido Poli (San Raffaele Scientific Institute, Milano, Italy) and Dr. Dan R. Littman (Skirball Institute of Biomolecular Medicine, New York).

#### Human DNA Constructs

Human cDNAs of the  $\beta$ -chemokine CCR1, CCR2b, CCR3, and CCR4 receptors were from OriGene (Origene Technologies, Inc., Rockville, MD).



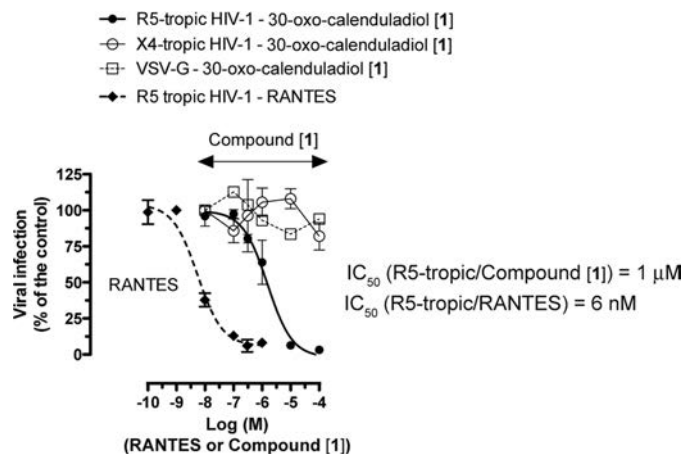
**FIGURE 2. 30-Oxo-calenduladiol (1) perturbs R5-tropic HIV-1 Env-mediated membrane fusion.** In *A* and *B*, quantification ( $\beta$ -galactosidase production) of Env-mediated cell fusion between HeLa-243 (X4-tropic Env) or HeLa-ADA (R5-tropic Env) cells and HeLa-P5 (CD4+/CCR5+/CXCR4+) cells in the absence (control) or presence of different lupane-type pentacyclic triterpenes (compounds 1–6) at 5  $\mu$ M. Compound 1 inhibited about 40% of the R5-tropic Env-mediated cell fusion process. A neutralizing anti-CD4 mAb (5  $\mu$ g/ml) was used to completely inhibit Env-mediated cell-to-cell fusion. The values are from three independent experiments (mean  $\pm$  S.E.;  $n = 9$ ).

#### Viral DNA Constructs

The pNL4-3.Luc.R-E- provirus (catalog number 6070013), the HXB2-env (catalog number 5040154), and pCAGGS-SF162-gp160-env (catalog number 3041817) glycoprotein vectors, and the pHEF-VSV-G vector (catalog number 4693), encoding the vesicular stomatitis virus G (VSV-G) protein, were obtained through the NIH AIDS Research and Reference Reagent Program.

#### HIV-1-Env-mediated Cell-to-cell Fusion Assay

A  $\beta$ -galactosidase cell fusion assay was performed as previously described (19, 20). Briefly, HeLa-243 or HeLa ADA cells were mixed with HeLa-P5 cells, in 96-well plates, in a 1:1 ratio (20,000 total cells), in the absence or presence of 5  $\mu$ M of the different molecules assayed. These co-cultures were kept at fusion for 16 h at 37 °C. The fused cells were washed with Hanks' balanced salt solution, lysed, and the enzymatic activity was evaluated by chemiluminescence ( $\beta$ -galactosidase reporter gene assay; Roche Diagnostics, Germany). Anti-CD4 neutralizing mAb (5  $\mu$ g/ml was preincubated in HeLa-P5 cells for 30 min at 37 °C before co-culture with Env+ HeLa cells) was used as a control for the blockage of cell fusion.



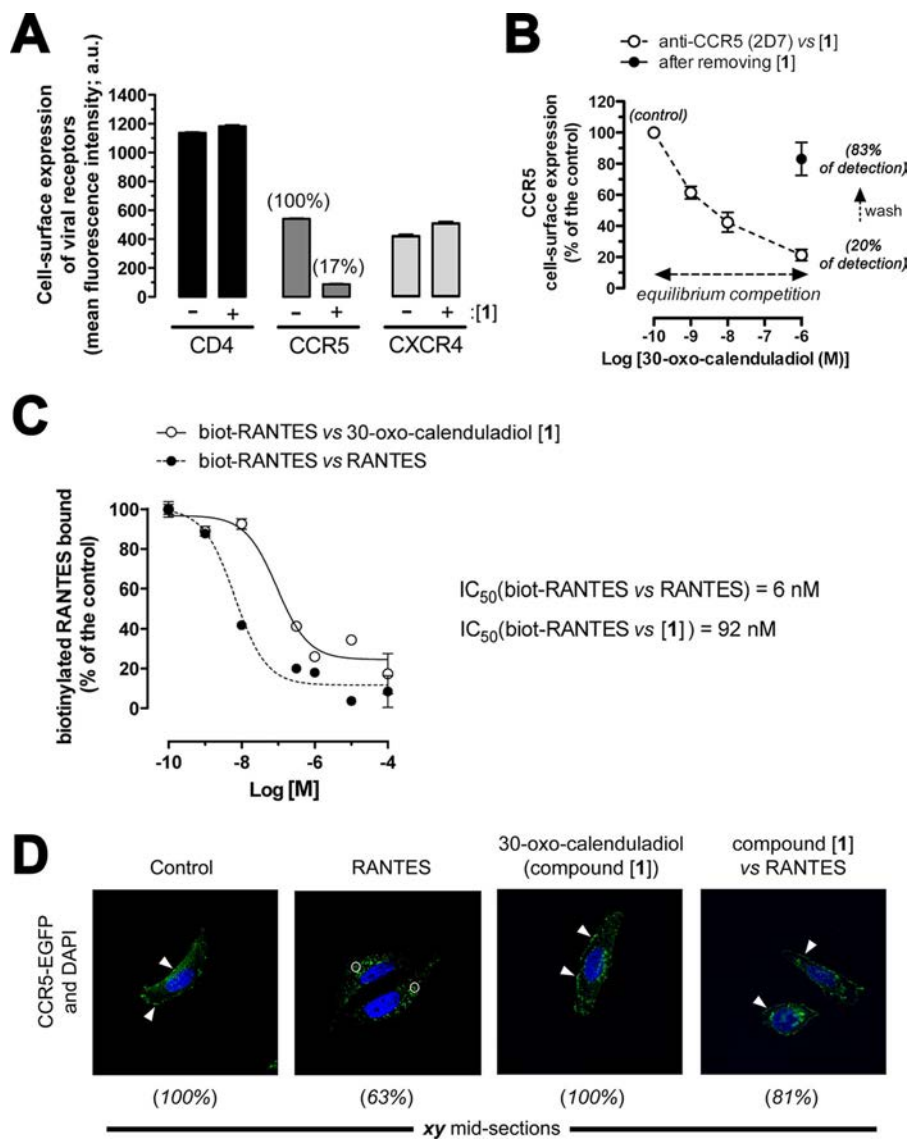
**FIGURE 3. 30-Oxo-calenduladiol (1) specifically inhibits R5-tropic HIV-1 infection.** CEM.NKR-CCR5 permissive cells were incubated with equivalent viral inputs, as determined by a standard p24-enzyme-linked immunosorbent assay test, of luciferase-based X4- or R5-tropic pNL4-3.Luc.R-E- virions. Then, cells were kept in culture for 2 days at 37 °C. To control the specificity of the compound 1-mediated effect on HIV-1 viral infection, luciferase-based VSV-G virions were used. Viral infected cells were determined by measuring the luciferase activity. Percentage inhibition of luciferase activity (of viral entry) was calculated for each dose point after subtracting the background (in the presence of a neutralizing anti-CD4 mAb at 5  $\mu$ g/ml), and  $IC_{50}$  was determined. Each assay was done in triplicate and results are representative of four independent experiments (mean  $\pm$  S.E.;  $n = 12$ ).

#### Production of Viral Particles

X4- or R5-tropic HIV-1 viral particles were produced by co-transfecting 293T cells (70% of confluence) in 75-cm<sup>2</sup> flasks with pNL4-3.Luc.R-E- (20  $\mu$ g) and CXCR4-tropic (HXB2-env) or CCR5-tropic (pCAGGS SF162 gp160) env glycoprotein (10  $\mu$ g) vector, as previously described (21). Co-transduction of the pNL4-3.Luc.R-E- (20  $\mu$ g) vector with the pHEF-VSV-G (10  $\mu$ g) vector generates non-replicative viral particles that infect with cells in a VSV-G-dependent manner. Viral plasmids were transduced in 293T cells by using linear polyethylenimine, with an average molecular mass of 25 kDa (PEI25k) (Polyscience Inc., Warrington, PA). For this purpose, viral plasmids were first dissolved in 1/10th of the final tissue culture volume of Dulbecco's modified Eagle's medium, free of serum and antibiotics. The PEI25k was prepared as a 1 mg/ml solution in water and adjusted to neutral pH. After addition of PEI25k to the viral plasmids (at a plasmids:PEI25k ratio of 1:5 (w/w)), the solution was mixed immediately, incubated for 20–30 min at room temperature and then added to 293T cells in culture. After 4 h the medium was changed to RPMI 1640, supplemented with 10% fetal calf serum and antibiotics, and the cells were cultivated to allow viral production. Viruses were harvested 40 h post-transfection. The supernatant was clarified by centrifugation at 3,000  $\times g$  for 30 min. Virions were then stored at –80 °C. Viral stocks were normalized by p24-Gag content measured with an enzyme-linked immunosorbent assay test (Innogenetics, Gent, Belgium).

#### Viral Infection Assay

$1 \times 10^6$  CEM.NKR-CCR5 permissive cells were incubated in the presence of different amounts of RANTES or 30-oxo-calenduladiol, with a synchronous dose of luciferase-based X4- or R5-tropic HIV-1 or VSV-G viral inputs (500 ng of p24), in 500  $\mu$ l of RPMI 1640 medium for 2 h, as described (21). Cells were then



**FIGURE 4. 30-Oxo-calenduladiol (1) interacts with the CCR5 receptor without promoting receptor internalization.** *A*, flow cytometry-based study of the ability of compound **1** ( $1 \mu\text{M}$ , for 1 h at  $4^\circ\text{C}$ ) to perturb the recognition of the CD4, CXCR4, and CCR5 viral receptors by their specific mAbs on the cell surface of CEM.NKR-CCR5 permissive cells. Compound **1** inhibits CCR5 recognition by a specific anti-CCR5 mAb (83% of inhibition). Data are from three independent experiments (mean  $\pm$  S.E.;  $n = 9$ ). *B*, equilibrium competition binding of the anti-CCR5 mAb 2D7 in the presence of different concentrations of compound **1** (open circles), for 1 h at  $4^\circ\text{C}$  in CEM.NKR-CCR5 cells. The solid circle indicated the recovery of the ability of the mAb 2D7 to recognize the cell surface CCR5 receptor at  $4^\circ\text{C}$ , after removing the pre-added compound **1** ( $1 \mu\text{M}$ ) by extensively washing cells at  $4^\circ\text{C}$ . *C*, competition binding assay. CEM.NKR-CCR5 cells were incubated with 5 nM human biotinylated RANTES and competed with different amounts of unlabeled human RANTES or 30-oxo-calenduladiol, for 1 h at  $4^\circ\text{C}$ . Incubations were terminated by centrifugation at  $4^\circ\text{C}$ , and cell pellets were resuspended in ice-cold PBS and the biotinylated RANTES bound was measured by flow cytometry after specific fluorescein isothiocyanate-avidin-mediated labeling. Nonspecific binding was determined in the presence of  $1 \mu\text{M}$  of unlabeled RANTES. Results are from six independent experiments (mean  $\pm$  S.E.;  $n = 18$ ). Non-linear analysis of the competition curves yielded  $\text{IC}_{50}$  binding values as follows: biotinylated RANTES versus RANTES (solid circles),  $6 \pm 0.23$  nM; biotinylated RANTES versus compound **1** (open circles),  $92 \pm 0.4$  nM. *D*, analysis of the 30-oxo-calenduladiol ( $1 \mu\text{M}$ , for 1 h at  $37^\circ\text{C}$ ) effect on CCR5-EGFP internalization in HeLa-P5 cells by confocal microscopy. RANTES ( $150$  nM, for 30 min at  $37^\circ\text{C}$ )-mediated CCR5-EGFP internalization is shown (see circled CCR5-EGFP receptors in the RANTES image) and its inhibition by compound **1** (in the presence of  $1 \mu\text{M}$ ; see white arrowheads in the 30-oxo-calenduladiol versus RANTES image). White arrowheads or circles indicate non-internalized or internalized CCR5-EGFP receptor in *xy* midsections, respectively. The green fluorescence monitors the stably transfected CCR5-EGFP receptor. 4',6-Diamidino-2-phenylindole-associated nuclei stain is shown. Percentages quantify the displayed pattern of CCR5-EGFP expression, RANTES-mediated internalization or compound **1**-dependent blockade from every 100 cells counted per each experimental condition. Data are from three independent experiments.

extensively washed to remove free virions. After 32 h of infection, luciferase activity was determined by using a luciferase assay kit (Promega Corporation) with a microplate

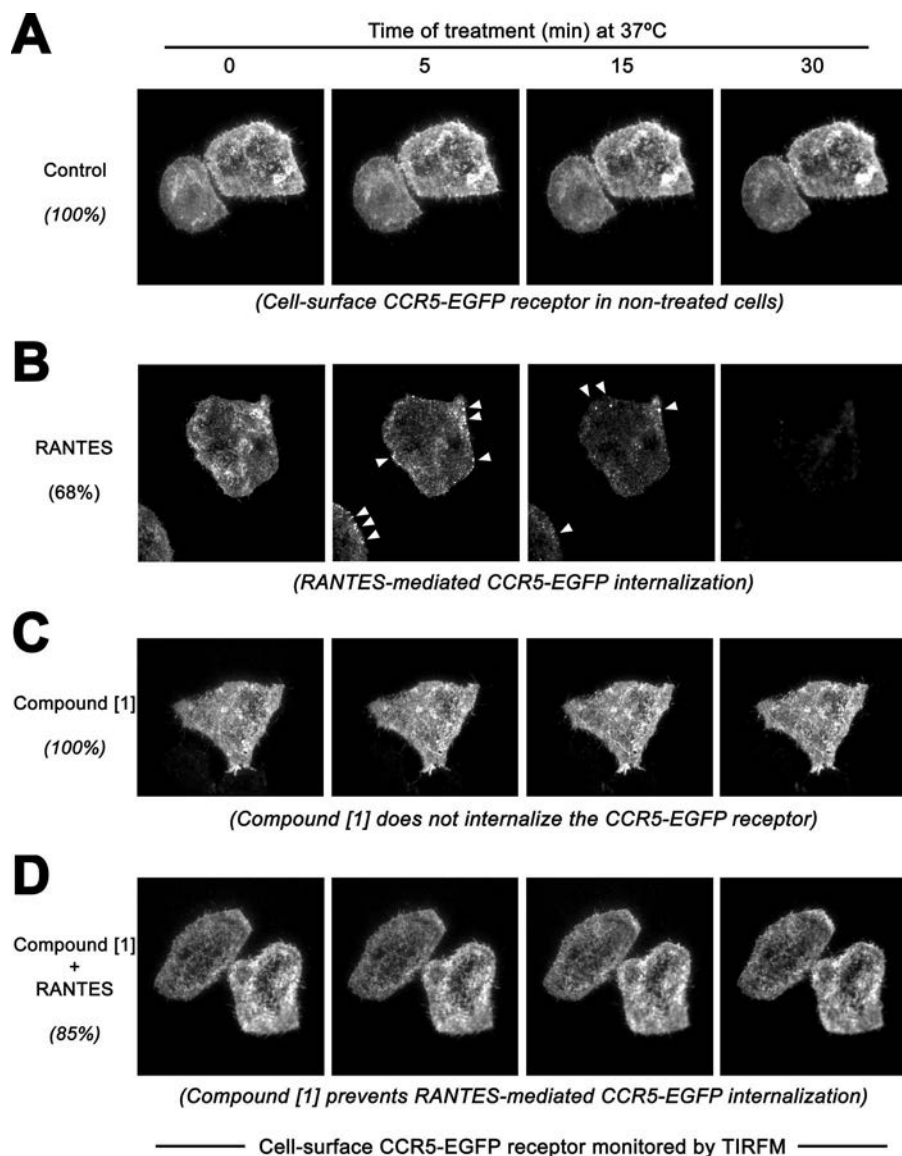
reader (GeNios<sup>TM</sup>, Tecan Trading AG, Switzerland). Data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA).

### Flow Cytometry Analysis

CEM.NKR-CCR5 cells were incubated with PE-labeled specific antibodies against CD4, CXCR4, or CCR5 in the presence of different amounts of the 30-oxo-calenduladiol compound, for 1 h at  $4^\circ\text{C}$  to avoid receptor internalization. Then, cells were washed by ice-cold PBS, fixed in PBS, 1% formaldehyde, and analyzed by flow cytometry (XL-MCL system, Beckman-Coulter Inc.). Cell surface expression analysis of CCR1, CCR2b, CCR3, or CCR4 receptors, transiently expressed in 293T cells, was similarly performed by using specific PE-conjugated mAbs, and their binding were equally assayed in the presence of 30-oxo-calenduladiol.

### Confocal Microscopy Analysis of CCR5 Internalization

HeLa-P5 cells on coverslips at 50% confluence were starved for 30 min at  $37^\circ\text{C}$  in Dulbecco's modified Eagle's medium without serum. They were then incubated in  $80 \mu\text{l}$  of Dulbecco's modified Eagle's medium (with 0.5% bovine serum albumin) with RANTES ( $150$  nM) or 30-oxo-calenduladiol ( $1 \mu\text{M}$ ) at  $37^\circ\text{C}$  for 30 min, or for 15 min at  $37^\circ\text{C}$  with 30-oxo-calenduladiol ( $1 \mu\text{M}$ ) before adding RANTES ( $150$  nM) for a further 30 min at  $37^\circ\text{C}$ . These CCR5-EGFP+ cells were rinsed three times at the end of incubation with ice-cold PBS and fixed in PBS, 2% paraformaldehyde for 3 min at room temperature. Cells were then rinsed and mounted in ProLong<sup>®</sup> Gold antifade reagent (Invitrogen) containing the 4',6-diamidino-2-phenylindole probe to stain the nucleus of cells. The internalization of the CCR5-EGFP molecule was ana-



**FIGURE 5.** 30-Oxo-calenduladiol (1)-mediated inhibition of RANTES-induced CCR5 internalization analyzed by TIRFM. TIRFM-based time-lapse study, in living HeLa-P5 cells at 37 °C, of CCR5-EGFP expression at plasma membrane of untreated (control) cells (A), cells treated with RANTES (150 nM) (B) or compound 1 (1 μM) (C), or previously treated with compound 1 (1 μM), for 15 min at 37 °C, before adding RANTES (150 nM) (D). In B, white arrowheads, in the 5- and 15-min images indicate clusters of CCR5-EGFP induced by RANTES, during receptor internalization. Percentages indicate the quantification of cells displaying the pattern of CCR5-EGFP expression (A, C, and D) or internalization (B) per each 100 cells counted, under any experimental condition. A representative experiment of three is shown.

#### Total Internal Reflection Fluorescence Microscopy (TIRFM) Analysis of CCR5 Internalization

HeLa-P5 cells, stably expressing the CCR5-EGFP receptor, were imaged with an inverted microscope Zeiss 200M (Zeiss, Germany) through a 1.45 NA objective ( $\alpha$ Fluar,  $\times 100/1.45$ ; Zeiss) in a Krebs-Hepes buffer containing 2 mM  $\text{Ca}^{2+}$  at 37 °C, in the absence or presence of RANTES (150 nM), compound 1 (1 μM), or pre-incubating compound 1 (1 μM), for 15 min at 37 °C, before adding RANTES (150 nM). RANTES-induced CCR5-EGFP internalization, or its prevention by compound 1 was analyzed by TIRFM technology, as described (22, 23). Briefly, total internal reflection generates an evanescent field (EF) that declines exponentially with increasing distance from the interface, depending on the

angle at which the light strikes the interface. The angle was measured using a hemicylinder, as described (24). The images were projected onto a back-illuminated CCD camera (AxioCam MRm, Zeiss) through a dichoric and specific band-pass filter for the EGFP fluorophor. CCR5-EGFP molecules were analyzed at 37 °C, and imaged on the cell surface of HeLa-P5 cells using Axiovision (Zeiss) with 0.5-s exposures at 10 Hz, when illuminated under EF at the indicated times for any experimental condition.

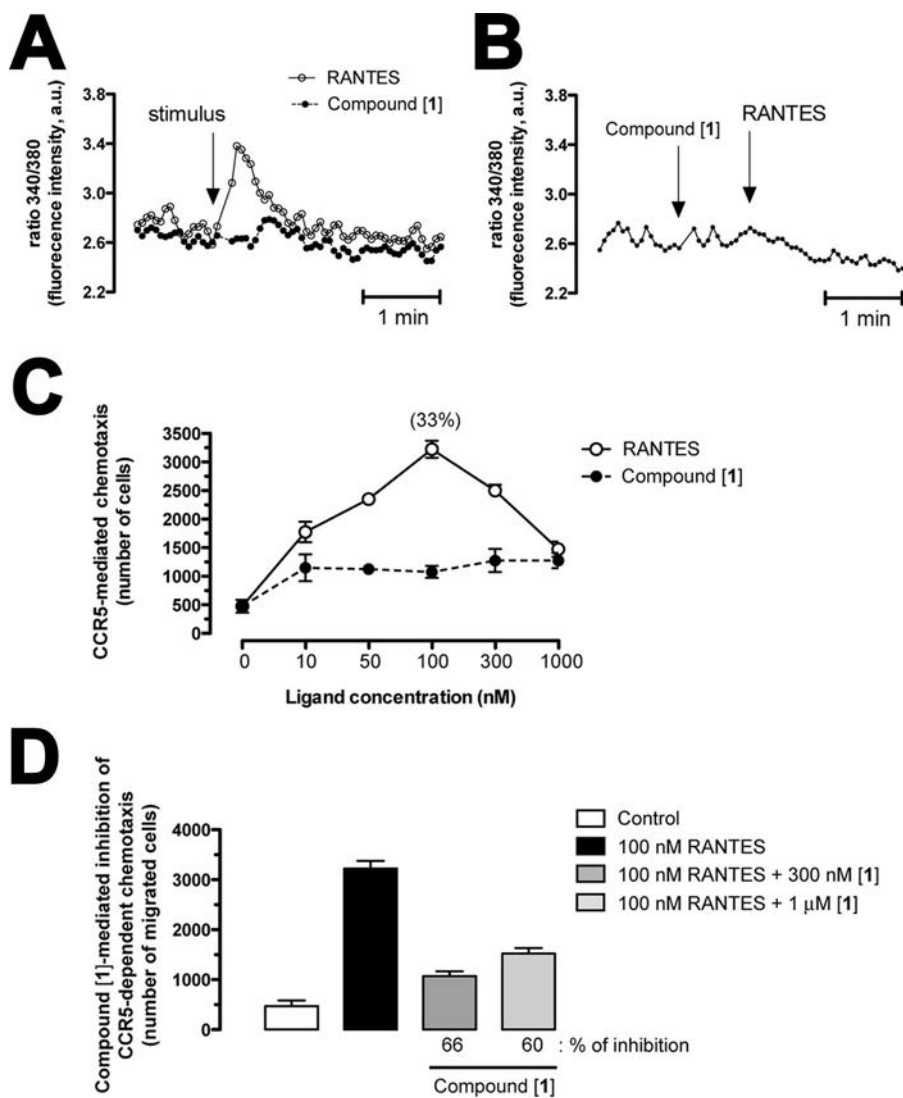
#### Ligand Competition Assays

CEM.NKR-CCR5 cells were incubated with 5 nM human biotinylated RANTES and competed with different amounts of unlabeled human recombinant RANTES, as indicated by the manufacturer (Fluorokine™ RANTES (CCL5) kit, R&D Systems), or the 30-oxo-calenduladiol triterpene in a final volume of 300 μl for 1 h at 4 °C. Incubations were terminated by centrifugation at 4 °C. Cell pellets were resuspended in ice-cold PBS and fluorescein isothiocyanate-labeled avidin (The Fluorokine™ biotinylated RANTES (CCL5) kit, R&D Systems) was added to detect bound biotinylated RANTES. The CCR5-associated biotinylated RANTES was quantified by flow cytometry. Binding assays for human CCR1, CCR2b, CCR3, or CCR4 receptor (OriGene), transiently transfected in 293T cells, were carried out in a similar way, by analyzing the ability of 30-oxo-calenduladiol to compete the specific binding of their respective biotinylated natural ligands

(RANTES, MCP-1, Eotaxin, or TARC (R&D Systems), respectively). The nonspecific binding of each ligand assayed was measured in cells pre-treated with specific blocking anti-receptor Abs (1 μg/ml) (included in the respective Fluorokine kits (R&D Systems)), which entirely prevented specific ligand-receptor binding.

#### Measurement of Cytosolic Free Calcium

Intracellular calcium levels were measured in a fluorescence spectrophotometer (Eclipse Variant; Melbourne, Australia) using Fura 2-AM (5 μM)-loaded CEM.NKR-CCR5 cells ( $5 \times 10^6$  cells/ml), as similarly described (25). Briefly, cells were resuspended in Hanks' balanced salt solution buffer (140 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{MgSO}_4$ ,



**FIGURE 6. 30-Oxo-calenduladiol (1) is a CCR5 antagonist that inhibits RANTES-mediated cell signal and cell chemotaxis.** *A*, intracellular calcium mobilization, a comparative analysis of RANTES versus compound **1** was performed using Fura 2-loaded CEM.NKR-CCR5 cells at 37 °C in a fluorescence spectrophotometer. 30-Oxo-calenduladiol did not induce calcium mobilization at any concentration tested (ranging from 5 nM to 1 μM), and the result obtained at 1 μM is shown. RANTES-mediated calcium mobilization at 150 nM is shown. A representative experiment of three is shown. *B*, intracellular calcium mobilization, a competitive analysis of compound **1** (1 μM)-mediated inhibition of RANTES (150 nM)-dependent calcium mobilization is shown, in Fura 2-loaded CEM.NKR-CCR5 cells at 37 °C. A representative experiment of three is shown. *C*, chemotaxis assay, migration of CEM.NKR-CCR5 lymphoblastoid cells was measured following stimulation with the indicated concentrations of RANTES or compound **1** for 3 h at 37 °C, and migrated viable cells were quantitated. The percentage at 100 nM RANTES indicates cells migrated with respect to the total cellular input. Results are from four independent experiments (mean ± S.E.;  $n = 12$ ). *D*, competitive chemotaxis assay, optimal RANTES (100 nM)-mediated migration of CEM.NKR-CCR5 cells was inhibited by compound **1** (at 300 nM and 1 μM) for 3 h at 37 °C. Results are from four independent experiments (mean ± S.E.;  $n = 12$ ).

1.2 mM CaCl<sub>2</sub>, 10 mM HEPES, 5 mM glucose, 0.3 mM KH<sub>2</sub>PO<sub>4</sub>, and 2 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, for 30 min at 37 °C. For calcium measurements, aliquots of this cell suspension were preincubated in a 1-ml cuvette, in a total volume of 200 μl (1 × 10<sup>6</sup> cells/ml) in Hanks' balanced salt solution supplemented with 5% heat-inactivated fetal calf serum, pH 7.4, for 5 min at 37 °C. RANTES or the 30-oxo-calenduladiol triterpene were added at the indicated times. The intracellular calcium mobilization was estimated by the 340:380 nm fluorescence ratio, per each experimental condition.

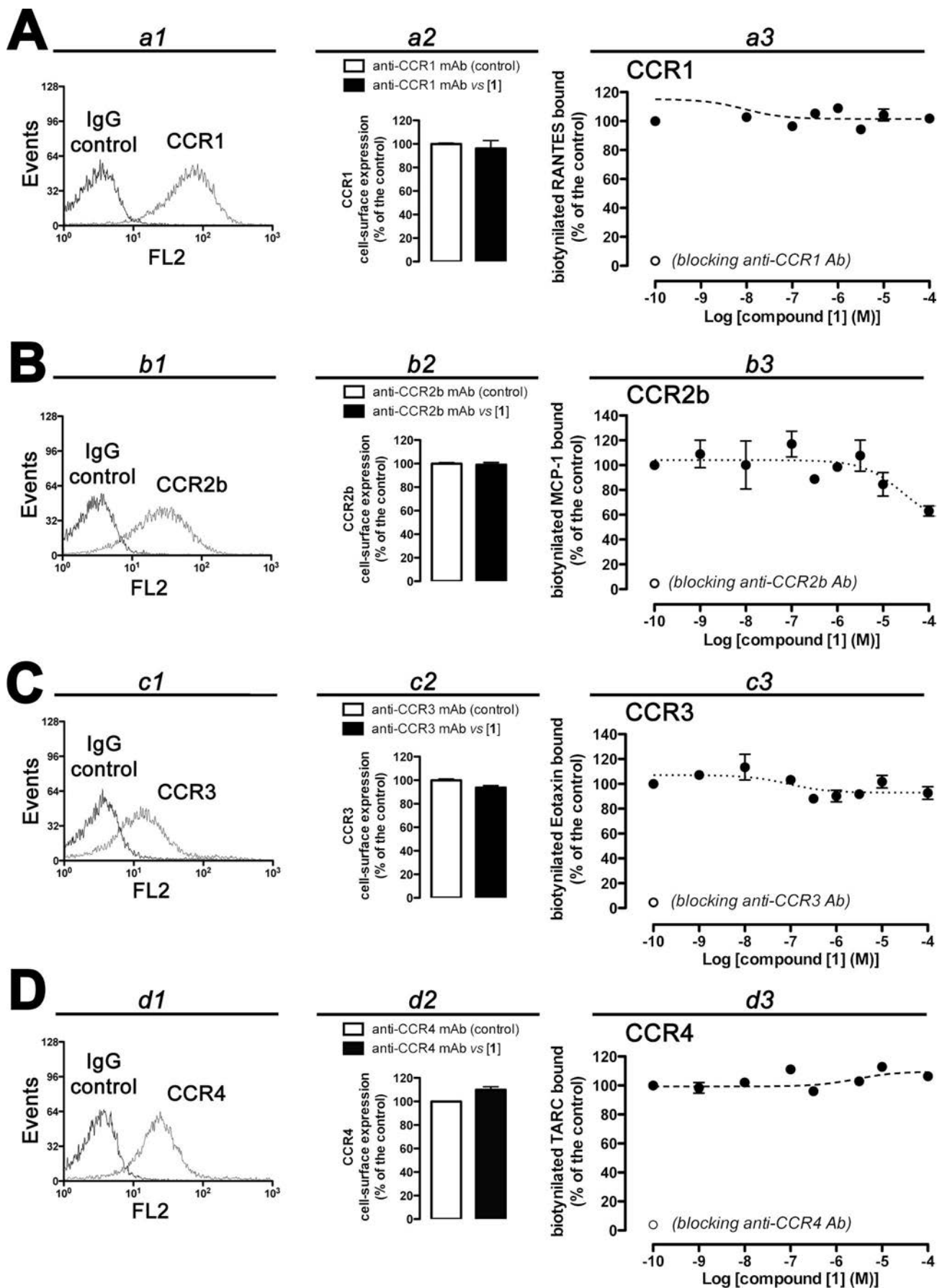
fusion (Fig. 2, compound **1**). The rest of the triterpene molecules analyzed did not present any anti-fusogenic activity against X4- or R5-tropic HIV-1-Env, in this HeLa-based cellular model (Fig. 2). None of the molecules assayed were toxic (ranging from 1 nM to 10 μM), as analyzed by flow cytometry and propidium iodide cellular labeling (supplemental Fig. S1). Hence, this data could suggest that the 30-oxo-calenduladiol triterpene may act specifically against the R5-tropic HIV-1 virus.

### Chemotaxis Assay

Migration of CEM.NKR-CCR5 cells was assessed in 48-well chambers (Neuro Probe Inc., Cabin John, MD), as previously described (25). Briefly, RANTES or 30-oxo-calenduladiol were added to the lower well at different concentrations, in a total volume of 30 μl in 25 mM HEPES-buffered RPMI 1640 at pH 7.4 (chemotaxis medium). The chemotaxis chamber was then assembled using polyvinylpyrrolidone-free polycarbonate membranes with 8-μm pore size (Costar, Cambridge, MA), and 50 μl of CEM.NKR-CCR5 cells (1 × 10<sup>6</sup> cells/ml), in chemotaxis medium without chemokine, was added to the upper well. After incubation in a 5% CO<sub>2</sub> humidified incubator at 37 °C for 3 h, the chamber was disassembled, and cells that migrated through to the lower wells were transferred to a working 96-well plate. The migrated viable cells were then quantified by flow cytometry.

### RESULTS

*The 30-Oxo-calenduladiol Triterpene Specifically Inhibits R5-tropic HIV-1 Infection*—We have studied a series of natural and semi-synthesized lupane-type triterpenes, with a similar backbone chemical structure (Fig. 1, compounds **1–6**), to identify new small molecules able to inhibit HIV-1 infection. First, the capacity of these triterpenes to perturb X4- and R5-tropic HIV-1-envelope (Env)-mediated fusion membrane was assayed in a cell-to-cell fusion model (19, 20). It was observed that the semi-synthetic 30-oxo-calenduladiol triterpene (**1**) perturbs R5-tropic Env-mediated cell-to-cell fusion, without affecting X4-tropic Env-mediated cell





Subsequently, the ability of 30-oxo-calenduladiol, and the others lupane-type triterpenes (Fig. 1) to inhibit HIV-1 viral infection was analyzed. We observed that 30-oxo-calenduladiol inhibited the infection of luciferase-based R5-tropic HIV-1 virions, in CCR5+/CXCR4+/CD4+ permissive cells (Fig. 3,  $IC_{50} = 1 \mu\text{M}$ ). However, this compound (**1**) did not inhibit cell infection by luciferase-based X4-tropic HIV-1 viral particles or by luciferase-based VSV-G fusogenic virions (Fig. 3). In this regard, the rest of triterpenes assayed (Fig. 1, *compounds 2–6*) did not present any antiviral activity against HIV-1 or VSV-G virions (data not shown). These data correlate with the results obtained in HIV-1-Env-mediated cell-to-cell fusion assays with the R5- and X4-tropic viral Envs (Fig. 2).

Thereby, it appears that 30-oxo-calenduladiol has specific antiviral activity, as the natural CCR5-ligand CCL5 (RANTES) (Fig. 3,  $IC_{50} = 6 \text{ nM}$ ), against R5-tropic HIV-1 virus, which infects permissive cells in a CD4/CCR5-dependent manner. 30-Oxo-calenduladiol was less effective than the chemokine RANTES in inhibiting HIV-1 infection, but it shows a good antiviral potency indicating that it could be considered as an interesting chemical structure to develop new anti-HIV-1 molecules. Moreover, the absence of any effect of the triterpene (**1**) on the infection of VSV-G viral particles suggests that 30-oxo-calenduladiol does not affect general viral-induced membrane fusion events, thereby specifically acting against R5-tropic HIV-1 virus.

**30-Oxo-calenduladiol Specifically Interacts with the CCR5 Chemokine Receptor**—To further analyze whether 30-oxo-calenduladiol (**1**) inhibits R5-tropic HIV-1 viral infection by directly interacting with CCR5, or some of the other viral receptors, binding of specific antibodies against the CD4, CCR5, or CXCR4 molecules was performed in CD4+/CCR5+/CXCR4+ permissive cells, in the presence of  $1 \mu\text{M}$  compound **1** for 1 h at  $4^\circ\text{C}$  (experimental conditions that avoid receptor internalization). Flow cytometry analysis showed that compound **1** impaired the binding of the neutralizing antibody (2D7) directed to CCR5, without affecting the antibody-mediated recognition of the CD4 or CXCR4 cell-surface antigen (Fig. 4A). Hence, compound **1** inhibited 2D7/CCR5 binding in a dose-dependent manner at  $4^\circ\text{C}$  (Fig. 4B, *open circles*). The CCR5/compound **1** interaction seems to be reversible, because the ability of compound **1** to compete the 2D7-CCR5 binding at  $4^\circ\text{C}$  (Fig. 4B, about 80% of inhibition at  $1 \mu\text{M}$  of **1**) was almost entirely reverted after extensively washing pre-treated cells at  $4^\circ\text{C}$  (Fig. 4B, *solid circle* indicates about 83% of 2D7-CCR5 binding).

To further confirm the ability of compound **1** to interact with the CCR5 chemokine receptor, we competed the binding of biotinylated RANTES to the CCR5 receptor at equilibrium with different concentrations of this lupane-type triterpene. It was

determined that compound **1** competed the biotinylated RANTES/CCR5 binding with an  $IC_{50}$  value of  $92 \text{ nM}$ , compared with the  $IC_{50}$  value of  $6 \text{ nM}$  for unlabeled RANTES (Fig. 4C). Hence, 30-oxo-calenduladiol appears to bind to CCR5 with an attractive affinity, quite similarly to RANTES. These data correlate with the specific neutralizing activity shown by this triterpene against R5-tropic HIV-1 Env (Figs. 2 and 3).

Therefore, it seems that compound **1** inhibits R5-tropic viral infection by interacting with the CCR5 viral co-receptor, without affecting cell surface CD4 or CXCR4 receptors on permissive cells. These data could explain the fact that compound **1** did not inhibit X4-tropic HIV-1 viral infection (Fig. 3).

**30-Oxo-calenduladiol Prevents RANTES-mediated CCR5 Internalization**—Despite the above data were obtained under experimental conditions that prevented the internalization of the CCR5 molecule, it is conceivable that compound **1** could promote the internalization of CCR5 during viral infection. Thus, the ability of this triterpene to internalize CCR5 was studied and compared with RANTES-mediated CCR5 internalization at  $37^\circ\text{C}$ , in permissive HeLa-P5 cells that stably express the fluorescent C-terminal EGFP-tagged CCR5 (CCR5-EGFP) receptor (Fig. 4D, in the *control* image, *white arrowheads* indicate cell-surface CCR5-EGFP). It was observed by confocal microscopy that RANTES induced the internalization of the cell-surface CCR5-EGFP receptor (Fig. 4D, see *circled* internalized CCR5-EGFP in the RANTES image), which was not observed at the cell surface of RANTES-treated cells. On the contrary, compound **1** did not internalize the chemokine receptor, even at concentrations of  $1 \mu\text{M}$  (Fig. 4D, see *white arrowheads* indicating CCR5-EGFP at the cell surface in the 30-oxo-calenduladiol image). Remarkably, RANTES-induced CCR5 internalization was impaired in the presence of 30-oxo-calenduladiol (Fig. 4D, see *white arrowheads* indicating CCR5-EGFP at cell surface in the 30-oxo-calenduladiol versus RANTES image).

To further support these data, we have analyzed by TIRFM the ability of 30-oxo-calenduladiol to impair RANTES-mediated CCR5 internalization, in living cells at  $37^\circ\text{C}$  (Fig. 5). The EF generated by TIRFM selectively illuminates fluorescent molecules near the plasma membrane interface, and leaves more remote cytosolic structures in the dark. Hence, the EF reaches from the plasma membrane into the cytosol for little more than  $100 \text{ nm}$ , allowing to monitor the dynamic behavior of molecules at plasma membrane (22–24), as ligand-induced internalization of the CCR5-EGFP molecule.

First, the steady-state distribution of CCR5-EGFP receptors on the cell surface of untreated HeLa-P5 cells was monitored (Fig. 5A, *control*). Then, we observed that the addition of RANTES ( $150 \text{ nM}$ ) induced the formation of CCR5-EGFP clus-

**FIGURE 7. 30-Oxo-calenduladiol (**1**) does not interact with the CCR1, CCR2b, CCR3, or CCR4  $\beta$ -chemokine receptors.** Flow cytometry analysis of cell surface expression of human CCR1-, CCR2b-, CCR3-, or CCR4-transfected receptors in 293T cells (A (a1 panel), B (b1 panel), C (c1 panel), or D (d1 panel), respectively). A representative experiment of three is shown. Equilibrium competition binding of specific mAbs directed against the CCR1, CCR2b, CCR3, or CCR4 chemokine receptors in the presence of compound **1** ( $1 \mu\text{M}$ ), for 1 h at  $4^\circ\text{C}$  (A (a2 panel), B (b2 panel), C (c2 panel), or D (d2 panel), respectively). Data are normalized from three different experiments ( $n = 9$ ). Competition binding assay, 293T cells, transiently transfected with CCR1, CCR2b, CCR3, or CCR4 receptors, were incubated with  $5 \text{ nM}$  of their respective and specific human biotinylated-natural ligands (RANTES, MCP-1, Eotaxin, or TARC, respectively) and competed with different amounts of compound **1**, for 1 h at  $4^\circ\text{C}$  (*solid circle curves*, in A (a3 panel), B (b3 panel), C (c3 panel), or D (d3 panel), respectively). Incubations were terminated and competition data were obtained as indicated in the legend to Fig. 4C. Nonspecific binding was determined in the presence of  $1 \mu\text{g/ml}$  of specific blocking antibodies against the related chemokine receptor (*open circles*, in A (a3 panel), B (b3 panel), C (c3 panel), or D (d3 panel), respectively). Results are from three independent experiments (mean  $\pm$  S.E.;  $n = 12$ ).

ters at the plasma membrane, during the first minutes of treatment (Fig. 5B, *white arrowheads*, in the 5- and 15-min images, indicate clusters of CCR5-EGFP), which then internalized as monitored by the dim of the CCR5-EGFP-associated fluorescence in the EF of HeLa-P5 cells (Fig. 5B, cell surface CCR5-EGFP receptors disappeared from the EF, after 30 min of treatment). However, compound **1** (1  $\mu\text{M}$ ) did not induce the clustering of CCR5-EGFP molecules at the plasma membrane, and did not internalize the chemokine receptor (Fig. 5C). Remarkably, pre-treatment of HeLa-P5 cells with compound **1** (1  $\mu\text{M}$ ), during 15 min at 37 °C, prevented RANTES (150 nM)-mediated clustering and further internalization of the CCR5-EGFP receptor, which did not disappear from the EF (Fig. 5D). Thereby, all these data suggest that compound **1** specifically interacts with cell-surface CCR5 without promoting receptor internalization, and impairing the action of its natural ligand, RANTES.

**30-Oxo-calenduladiol Is a Specific CCR5 Antagonist**—To further investigate the actions of compound **1** on the normal function of the CCR5 receptor, it was assayed whether this molecule acts as an antagonist of this chemokine receptor. We observed that RANTES, a natural ligand of CCR5, triggered the mobilization of intracellular calcium (Fig. 6A), unlike compound **1**, which was not able to mobilize intracellular calcium at any concentration assayed in CCR5+ permissive cells (Fig. 6A, 1  $\mu\text{M}$  30-oxo-calenduladiol is only shown). Remarkably, preincubation of CCR5+ cells with compound **1**, before adding the chemokine ligand, impaired RANTES-mediated intracellular calcium mobilization (Fig. 6B). Thereby, it appears that compound **1** competed the binding and signaling of RANTES via the CCR5 receptor.

Moreover, the ability of compound **1** to induce CCR5-mediated cell migration and alter the chemotactic activity of RANTES was also studied. RANTES efficiently mediated chemotaxis of CCR5-expressing lymphocytes with a peak of chemotactic activity at 100 nM (Fig. 6C). On the contrary, 30-oxo-calenduladiol did not promote cell migration of these CCR5-expressing cells (Fig. 6C), and inhibited optimal CCR5-dependent chemotaxis mediated by RANTES (Fig. 6D).

To determine whether the inhibitory effect of 30-oxo-calenduladiol triterpene on chemokine binding is specific to CCR5 or is promiscuous to other  $\beta$ -chemokine receptors (26), the activity of compound **1** was examined in CCR1, CCR2b, CCR3, or CCR4 receptors, transiently expressed in 293T cells (Fig. 7, A (*a1 panel*), B (*b1 panel*), C (*c1 panel*), or D (*d1 panel*), respectively). We first observed that compound **1** did not affect the ability of some mAbs to recognize their specific human receptors, CCR1, CCR2b, CCR3, or CCR4, (Fig. 7, A (*a2 panel*), B (*b2 panel*), C (*c2 panel*), or D (*d2 panel*), respectively). Remarkably, compound **1** did not have any effect on the binding of RANTES, Eotaxin, or TARC to CCR1, CCR3, or CCR4, respectively (Fig. 7, A (*a3 panel*), B (*b3 panel*), C (*c3 panel*), or D (*d3 panel*), respectively). Regarding the CCR2b receptor, compound **1** slightly affected the binding of MCP-1, but only at high triterpene concentrations (Fig. 7B (*b3 panel*), 20% reduction of the total MCP-1 binding at 100  $\mu\text{M}$  compound **1**), and without signal (data not shown). Hence, our results indicate that compound **1** could not be considered as a potential ligand of CCR2b

(Fig. 7B (*b3 panel*)). Moreover, compound **1**-mediated equilibrium binding competition for the MCP-1/CCR2b or Eotaxin/CCR3 pairs was also assayed in the THP-1 monocytic cell line or U87.CD4.CCR3 cells, respectively, obtaining similar results in all cases (data not shown). Thereby, it seems that compound **1** is a selective antagonist for CCR5 (Figs. 2–6) and appears not to have any effect on CCR1, CCR2b, CCR3, and CCR4 chemokine receptors (Fig. 7). Altogether these data suggest that 30-oxo-calenduladiol behaves as a specific CCR5 antagonist to inhibit CCR5-mediated intracellular calcium mobilization and cell migration, presenting neutralizing activity against R5-tropic HIV-1 virus *in vitro*.

## DISCUSSION

In the present work we have described for the first time the anti-HIV-1 activity of 30-oxo-calenduladiol, compound **1**, a semi-synthetic dihydroxylated lupane-type triterpene. This compound specifically interfered with cellular fusion and infection mediated by R5-tropic viral Env and HIV-1 virus, respectively. Hence, compound **1** did not perturb the related processes mediated by X4-tropic HIV-1 virus or viral Env. This specific antiviral activity of compound **1** relies on its ability to interact with the HIV-1 co-receptor CCR5. This lupane-type triterpene seems not to interact with the CD4 antigen, the main viral receptor, or the CXCR4 co-receptor. Furthermore, compound **1** impaired the binding of CD195 (2D7), a specific anti-CCR5 mAb, to the CCR5 chemokine receptor, and competed with high affinity CCL5/CCR5 interaction.

The ability of compound **1** to compete the binding of the neutralizing anti-CCR5 mAb (2D7) or the natural ligand RANTES, and to inhibit R5-tropic HIV-1 infection may relay on its capacity to interact with the second extracellular loop of the CCR5 receptor. This region represents the conformational binding domain for the neutralizing antibody 2D7 (27), which has been involved in ligand/ or virus/receptor interaction (27–30). However, as occurs with other antiviral small molecules (31–34), it is also plausible that compound **1** may occupy the CCR5 pocket, inducing a conformational change that may affect the second extracellular loop of CCR5, impairing its recognition by the anti-CCR5 mAb CD195 (2D7). These potential conformational changes may also affect RANTES-CCR5 binding and CCR5/gp120-mediated R5-tropic HIV-1 viral infection.

In the course of HIV infection *in vivo*, R5-tropic HIV-1 viruses predominate in early asymptomatic stages of infection, whereas R5/X4-dual tropic and X4-tropic HIV-1 viral strains appear at later stages of HIV-1 infection (35). Furthermore, R5 viruses are responsible for transmission of HIV-1 as evidenced by the high degree of resistance to infection of individuals homozygous for a 32-bp deletion in the gene encoding CCR5, who consequently lack a functional receptor (36, 37). In this regard, it is thought that the blockade of R5-tropic HIV-1 infection could be key for preventing primary viral infection and transmission. Hence, the development of CCR5 antagonists is important to battle HIV viral infection, and to complement the existing antiretroviral strategies against AIDS in the near future. Because CCR5  $\Delta 32/\Delta 32$  homozygote persons exhibit no consequences of being CCR5 negative, and present a high degree of resistance against viral infection (36, 37), the discov-

ery of new CCR5 antagonists, which do not trigger cell signals or mediate immune responses, represents an emerging and important area of research (38–40).

In this regard, the 30-oxo-calenduladiol-associated  $IC_{50}$  value for the inhibition of R5-tropic HIV-1 infection was of 1  $\mu M$ . This  $IC_{50}$  value could be compared with those values obtained with good anti-HIV-1 molecules recently described (reviewed in Ref. 35).

Our results suggest that the 30-oxo-calenduladiol molecule is a non-toxic, specific CCR5 antagonist, which binds to the CCR5 receptor without triggering intracellular calcium mobilization or receptor internalization, and inhibits RANTES-mediated intracellular calcium mobilization, cell chemotaxis, and CCR5 internalization. Therefore, 30-oxo-calenduladiol-mediated anti-HIV-1 activity against the R5-tropic virus appears to rely in the occupancy of the CCR5 receptor to inhibit CCR5-mediated HIV-1 infection. Moreover, compound **1** also appears not to interact with other  $\beta$ -chemokine receptors, such as CCR1, CCR3, and CCR4. In addition, compound **1** shows a small degree of interaction with CCR2b, only at high concentrations (100  $\mu M$ ) of the molecule. It is plausible that the homology existing between CCR5 and CCR2b receptors (about 72% sequence identity) (reviewed in Ref. 41), may be responsible for the small degree of competition observed against the MCP-1/CCR2b binding. Altogether these results indicate that compound **1** is not a promiscuous antagonist for the above  $\beta$ -chemokine receptors, presenting selectivity of action on the CCR5 receptor.

Although target specificity is desired and required for antagonist-mediated treatment of several diseases, recently much expectation has been set upon the use of promiscuous, or polypharmacology compounds (42) to develop more efficient therapeutical approaches to battle multifactorial diseases (26, 41, 43, 44). Thereby, it would be interesting to further explore the potential antagonist activity of compound **1** on other cell surface receptors, different or not from the chemokine family.

Observing the structure of the 30-oxo-calenduladiol (**1**), and the structure of the different lupane-type triterpene assayed (Fig. 1), it is plausible that the two hydroxyl residues at carbons C-3 and C-16 together with the  $\alpha,\beta$ -unsaturated aldehyde function, at carbon C-30, could stabilize the 30-oxo-calenduladiol/CCR5 association by establishing hydrogen bonds with some complementary residues in the viral receptor pocket. Thereby, these functional groups may be responsible for the antiviral activity of the molecule, acting as Michael acceptors with some nucleophilic residues of the corresponding CCR5 viral receptor that would explain the specific activity against R5-tropic viral Env. Hence, compound **4** (Fig. 1), which possesses the two hydroxyl residues but not the aldehyde function, was inert against HIV-1 infection. Therefore, the chemical structure of the 30-oxo-calenduladiol triterpene could represent a good model to develop more potent anti-HIV-1 molecules. In this matter, compound **1** is a dihydroxylated ( $3\beta,16\beta$ -diol) lupane, containing a formyl group at position C-30 conjugated with a double bond. Interestingly, the betulinic acid, a pentacyclic triterpene with described anti-HIV-1 activity, is easily derived by the abundant naturally occurred betulin, a related lupane-type triterpene diol ( $3\beta,28$ -diol) (10). Hence, it is conceivable that

other natural lupane-type triterpene diols, as the 30-oxo-calenduladiol compound characterized in the present work, may present good anti-HIV-1 activities.

Considering all the presented data, we suggest that 30-oxo-calenduladiol is a new and specific CCR5 antagonist that exhibits great promise as a bioactive agent for the development of new high active derivatives for treatment of HIV-1 infection, and against biological disorders related to CCR5-mediated cell migration, such as for instance, inflammation, immune response, and tumor cell migration (45–57).

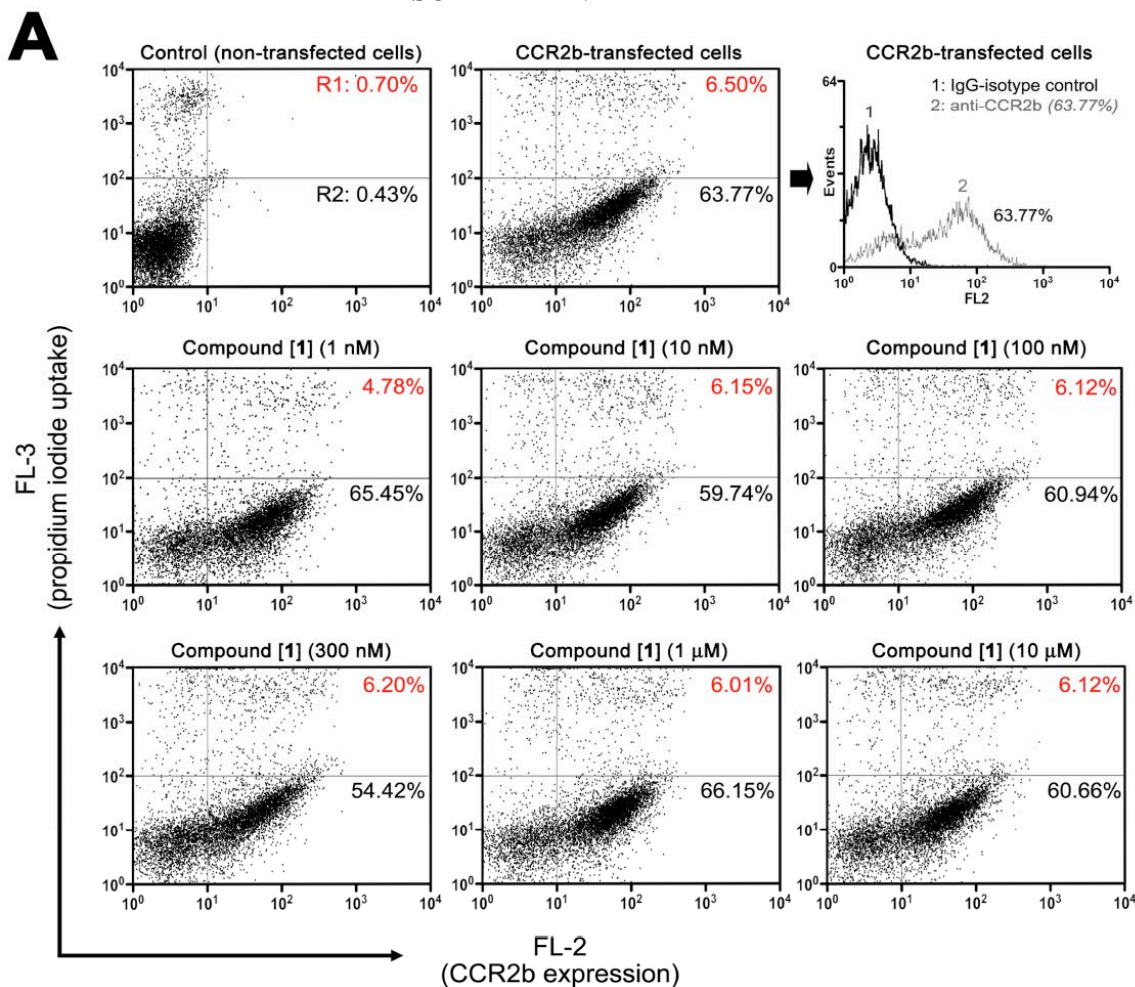
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## SUPPLEMENTAL DATA



Supplemental Figure 1  
Barroso-González et al.

### Legend to Supplemental Figures.

**Supplemental Figure 1. The different concentrations used of the lupane-type triterpene 30-oxo-calenduladiol [1] are not toxic for cells.**

A, flow cytometry-based analysis of propidium iodide uptake in control, non-transfected 293T cells (*top left panel*) and CCR2b-transfected 293T cells (24 h post-transfection) (*top middle and right panels, CCR2b-transfected cells*), or CCR2b-transfected 293T cells (24 h post-transfection) further treated by different concentrations of compound [1] (*from 1 nM to 10 μM*), for 6 h at 37°C. Quantification of propidium iodide uptake (FL3) by cells is indicated in regions 1 (R1, in red) of plots, per each experimental condition. The percentage of CCR2b-transfected 293T cells is indicated in regions 2 (R2, in black) of each plot. Cell-surface expressed CCR2b receptor is detected by using a specific PE-conjugated mAb (FL2), under any experimental condition. A representative experiment of three is shown.