Anti-HIV Activity and Resistance Profile of the CXC Chemokine Receptor 4 Antagonist POL3026

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We have studied the mechanism of action of Arg*-Arg-Nal2-Cys(1/H11003)-Tyr-Gln-Lys-(D-Pro)-Pro-Tyr-Arg-Cit-Cys(1/H9251)-Tyr-Gly-(D-Pro)*(POL3026), a novel specific β-hairpin mimetic CXC chemokine receptor (CXCR)4 antagonist. POL3026 specifically blocked the binding of anti-CXCR4 monoclonal antibody 12G5 and the intracellular Ca2+ signal induced by CXC chemokine ligand 12. POL3026 consistently blocked the replication of human immunodeficiency virus (HIV), including a wide panel of X4 and dualtropic strains and subtypes in several culture models, with 50% effective concentrations (EC50) at the sub-nanomolar range, making POL3026 the most potent CXCR4 antagonist described to date. However, 1-[[4-(1,4,8,11-tetrazacyclotetradec-1-ylmethyl)phenyl][methyl]-1,4,8,11-tetrazacyclotetradecane (AMD3100)-resistant and stromal cell-derived factor-1α-resistant HIV-1 strains were cross-resistant to POL3026. Time of addition experiments and a multiparametric evaluation of HIV envelope function in the presence of test compounds confirmed the activity of POL3026 at an early step of virus replication: interaction with the coreceptor. Generation of HIV-1 resistance to POL3026 led to the selection of viruses 12- and 25-fold less sensitive and with mutations in gp120, including the V3 loop region. However, POL3026 prevented the emergence of CXCR4-using variants from an R5 HIV-1 strain that may occur in the presence of anti-HIV agents targeting CC chemokine receptor 5.

HIV-1 particles require the interaction with CD4 receptor through gp120 and a coreceptor, mainly CCR5 or CXCR4 (Deng et al., 1996; Doms, 2001), to trigger fusion of viral and cellular membranes and entry into cells. HIV-1 variants can be classified into those that exclusively use CCR5 (R5 or CCR5-tropic viruses), CXCR4 (X4 or CXCR4-tropic viruses), or both coreceptors (R5X4 or dualtropic viruses) (Berger et al., 1998). The coreceptor use is determined by the amino acid sequence of HIV gp120, in particular the number and position of basic residues in the V3 and V1/V2 loops, and less frequently in other regions (Menéndez-Arias and Esté, 2004). Over the course of the infection, the coreceptor use of HIV changes from CCR5 to CXCR4 in 50% of infected individuals (Koot et al., 1993). The switch from R5 to X4 viruses is associated with accelerated CD4+ T-cell decline and progression to AIDS, but the mechanisms leading to the emergence of X4 variants are not fully understood.

Current antiretroviral treatment has reduced morbidity and mortality in HIV-1-infected individuals. However, it can-
not eradicate the virus from infected individuals, and it is often limited by the emergence of drug-resistant HIV-1 strains and long-term toxicity. These problems highlight the need to develop new anti-HIV-1 drugs targeting different steps in the viral replication cycle, such as the HIV entry process (Esté, 2003). In fact, a large number of compounds targeting different steps, structurally and temporally separated, have been developed, particularly the chemokine co-receptors antagonists are the most promising entry inhibitors. At least one anti-HIV agent targeting CCR5 has been approved for treatment of drug-experienced patients, and others are in advanced clinical trials (Esté and Telenti, 2007). HIV-1 may escape CCR5 treatment by the emergence of mutations that confer resistance in the absence of virus coreceptor switch. However, R5 strains may switch virus coreceptor in the presence of CCR5 inhibitors (Lalezari et al., 2007), or treatment with CCR5 inhibitors may select for minor populations of viruses with the ability to use CXCR4 (Westby et al., 2006).

Bicyclams are the first class of CXCR4 agents described to block HIV replication (Esté et al., 1999a; De Clercq, 2000, 2003), but a number of different agents, including polyphemusin II, have been shown to block X4 HIV-1 replication (Murakami et al., 1997), and some have progressed to clinical trials. The latest, AMD11070 (or AMD070) (Stone et al., 2007), was evaluated in a pilot monotherapy study with patients harboring X4 or R5/X4 viruses (Moyle et al., 2007), but it was suspended after liver histology changes and liver and retinal toxicity were observed in animal research studies. However, a greater than 1 log reduction of X4 was observed in four of nine patients, and three of the four responders switched from dual/mix virus to R5, proving proof of concept that CXCR4 antagonists can inhibit CXCR4-using viruses in vivo. There is a need for developing new potent CXCR4 antagonists with a safety profile suitable for human clinical use.

Highly potent and selective β-hairpin mimetic CXCR4 inhibitors with good pharmacokinetic profiles have been described (DeMarco et al., 2006). One of them, POL3026, has been chosen for further characterization in CXCR4 specificity, anti-HIV activity, and mode of action. POL3026 (mol. wt. 2114) was designed starting from a truncated analog of polyphemusin II. Some residues were changed giving a precursor from which a macrocyclic structure was generated by linking the N- and the C-terminal residues. Libraries of such peptidomimetics were synthesized by the Service of Peptide Synthesis (University of Barcelona, Barcelona, Spain) density gradient of buffy coats obtained from donors were isolated by separation on Ficoll-Hypaque (Atom Reagents, Barcelona, Spain). Aliquots (1 ml) were frozen and conserved, until needed, in liquid nitrogen. Monocytes and primary CD4+ T cells were purified from peripheral blood mononuclear cells by negative selection (Stem Cell Technologies, Vancouver, BC, Canada). Macrophages were obtained as described previously (Bosch et al., 2006). We cultured monocyes for 3 days with macrophage–colony-stimulating factor (Peprotech, London, UK) at 20 U/ml (100 ng/ml) at 50,000 cells/well in 96-well plates for viability and acute infection.

**Compounds.** The synthesis, purification, and chemical characterization of POL3026 (Fig. 1) were performed as described previously (DeMarco et al., 2006). The chemokine SDF-1α (natural ligand of CXCR4) and the natural ligands of CCR5 MIP-1α, MIP-1β, and RANTES were purchased from Peprotech (London, UK). The RT inhibitor 3-azido-3-deoxythymidine (zidovudine; AZT) was purchased from Sigma-Aldrich, and the oligonucleotide targeting gp120, zintevir (AR177), the CXCR4 antagonists AMD3100 and ALX-40-4C, and the fusion inhibitor peptide C34 were synthesized as described previously (Doran et al., 1997; Murakami et al., 1997; Esté et al., 1998, 1999a; Armand-Ugón et al., 2003a). CCR5 antagonist TAK-779, reviewed in Esté (2003), and the RT inhibitors efavirenz, nevirapine, and lamivudine (3TC), reviewed in De Clercq (2004), were received from the National Institutes of Health AIDS Reagent Program, and the monoclonal antibody anti-CCR5 PRO140 was from Progenics Inc. (Tarrytown, NY), reviewed in Lederman et al. (2006). The fusion inhibitor T-20 (enfuvirtide) (Melby et al., 2006) was synthesized by the Service of Peptide Synthesis (University of Barcelona, Barcelona, Spain).

**Viruses.** The HIV-1 strains BaL, HXB2, NL4-3, and 89.6 were obtained from the Medical Research Council Centre for AIDS Reagents. The X4 strain J130.3 was kindly provided by Dr. O. T. Keppler (University of Heidelberg, Heidelberg, Germany). The HIV-1 NL-4-3 strain that is resistant to T-20/C34 has been described previously (Armand-Ugón et al., 2003a; Menéndez-Arias and Esté, 2004). The IRLL98 HIV-1 strain contains the following mutations in the RT coding sequence: M41L, D67N, Y181C, M184V, R211K, and T215Y (conferring resistance to nucleoside reverse transcriptase inhibitors) and mutations K101Q, Y181C, and G190A (conferring resistance to non-nucleoside reverse transcriptase inhibitors). HIV-1 strains K103N, Y181C, and Y188L, which have mutations conferring resistance to non-nucleoside reverse transcriptase inhibitors, and HIV-2 ROD were obtained from the Medical Research Council Centre for AIDS Reagents. The AMD3100-resistant strain and the SDF-1α-resistant strain were derived after sequential passage of the NL4-3 virus in the presence of increasing concentrations of AMD3100 or SDF-1α in MT-4 cells (de Vreese et al., 1996). The X4 HIV-1 clinical isolate CI1, and the dualtropic CI2, CI3, and CI4 were selected in four of nine patients, and three of the four respond-

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**Materials and Methods**

**Cell Lines.** CD4+ lymphoid cell lines MT-4, THP-1, Sup-T1, and MT-2 were obtained through the Medical Research Council Centre for AIDS Reagents (London, UK). The human astrocytoma cell line U87 expressing CD4 and either CCR5 or CXCR4, the human T-lymphoblastoid A3.01/CCR5-A5 and F7 (CEM/CCR5), and MOLT-4/ CCR5 cell lines were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD). MT-4, THP-1, Sup-T1, and MT-2 cells were grown in RPMI 1640 medium, and U87 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Barcelona, Spain), supplemented with 10% fetal calf serum (FCS; Cambrex, Barcelona, Spain) and antibiotics 2 U/ml penicillin and 2 μg/ml streptomycin (Invitrogen). MOLT-4/ CCR5 cells chronically infected with an X4 isolate, NL4-3 or CI-1,SI, or the R5 isolate BaL were generated in our laboratory (Blanco et al., 2005). Peripheral blood mononuclear cells (PBMC) from six healthy donors were isolated by separation on Ficoll-Hypaque (Atom Reactiva, Barcelona, Spain) density gradient ofuffy coats obtained from the Catalonia Bank de Sang i Teixits (Barcelona, Spain). PBMC from each donor were mixed equally and resuspended at 50 × 10⁶ PBMC/ml in heat-inactivated FCS containing 10% dimethyl sulfoxide (Sigma-Aldrich, Madrid, Spain). Aliquots (1 ml) were frozen and conserved, until needed, in liquid nitrogen. Monocytes and primary CD4+ T cells were purified from peripheral blood mononuclear cells by negative selection (Stem Cell Technologies, Vancouver, BC, Canada). Macrophages were obtained as described previously (Bosch et al., 2006). We cultured monocytes for 3 days with macrophage–colony-stimulating factor (Peprotech, London, UK) at 20 U/ml (100 ng/ml) at 50,000 cells/well in 96-well plates for viability and acute infection.

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obtained by coculturing PBMC from HIV-1-infected patients with stimulated PBMC from healthy donors. HIV-1 168.1 is a R5 molecular clone virus (Moncunill et al., 2008).

**Anti-HIV and Cytotoxicity Assays.** Anti-HIV activity and cytotoxicity measurements in MT-4 cells were based on viability of cells that had been infected or not infected with HIV-1 at a multiplicity of infection of 0.003, and they were exposed to various concentrations of the test compound. After 5 days of infection, the number of viable cells was quantified by a tetrazolium-based colorimetric method (MTT method) as described previously (Armand-Ugón et al., 2003a, 2005). Anti-HIV activities were performed three times in triplicates. Fifty percent effective concentrations (EC50) were calculated as valid when the variation between replicates was less than 4-fold. Mean EC50 ± S.D. for control compounds AZT and AMD3100 was 0.0008 ± 0.0002 and 0.001 ± 0.0004 μg/ml, respectively. Cut-off value in which a virus was considered resistant was greater than 4-fold increase of the EC50 value compared with the wild type HIV-1 strain.

Anti-HIV activity in PMBC was determined as described previously (Moncunill et al., 2005). In brief, cells were incubated with each HIV-1 viral stock (200 50% tissue culture infectious dose/10⁶ cells) or mock-infected for 3 h at 37°C. Thereafter, they were washed twice with 1× phosphate-buffered saline (PBS). Infected cells were seeded in 96-well plates (0.15 × 10⁶ cells/well), and they were incubated 7 days at 37°C, 5% CO2 at different concentrations of the test compound in triplicates. HIV-1 p24 antigen production in the supernatant was measured by a commercial ELISA test (Innotest HIV-Ag; Innogenetics, Barcelona, Spain). To determine cytotoxicity, the mock-infected cells were harvested and fixed with 1% formaldehyde, 1× PBS. Cell death was quantified by flow cytometry in forward versus side scatter plots, as described previously. Dead cells showed increased side and reduced forward scatter values compared with those of living cells. Anti-HIV activities in PMBC were performed three times. To ensure the reproducibility of the assay, the mean EC50 and S.D. values were calculated for the control drugs AZT and AMD3100 against the NL4-3 wt strain. The EC50 was 0.001 ± 0.0005 μg/ml (n = 3) and 0.006 ± 0.001 μg/ml (n = 3) for AZT and AMD3100, respectively.

The antiviral assay in monocyte-derived macrophages (MDM) was carried after 3 days of stimulation with macrophage–colony-stimulating factor of monocytes. Cells were washed and incubated in complete culture medium containing various anti-HIV drugs. MDM were infected with the X4 HIV-1 strain J130.3 or with the R5 strain BaL at a final concentration of 3700 pg/ml HIV p24 antigen. At days 3, 7, 10, and 14 after infection, 20 μl of culture supernatant was replaced by 20 μl of fresh complete medium with or without the corresponding drug. HIV production was analyzed at days 7 and 14 after infection by HIV p24 antigen detection in the culture supernatants (Innotest HIV-Ag; Innogenetics).

Anti-HIV activity in lymphoid tissue was evaluated as described previously (Glushakova et al., 1998; Grivel and Margolis, 1999). Tonsils from HIV-negative individuals from therapeutic tonsillectomy were maintained in 1× PBS, dissected into 2- to 3-mm blocks, and placed on top of gelatin sponge gels (Espongostar; Prisfar, Porto, Portugal) with RPMI 1640 medium,10% FCS, and penicillin/streptomycin. HIV-1 infection was carried out with 1.5 μl of the R5 HIV-1 BaL, the X4 NL4-3, and the dualtropic HIV-1 89.6 p24-antigen supernatant in the absence or presence of the test compounds (AZT, AMD3100, TAK-779, and POL3026). Ten days after infection, the concentration of p24 antigen in the supernatant was evaluated by a commercial ELISA test (Innotest HIV-Ag; Innogenetics).

**Evaluation of Cell Death and HIV Transfer.** Primary CD4 T cells (2 × 10⁶) were cocultured with 2 × 10⁶ MOLT-4/CCR5 cells chronically infected with the X4 isolates NL4-3 and CI-1-8F. After 24 h of coculture, cells were washed with 1× PBS, fixed, and permeabilized (Fix and Perm; Invitrogen, Burlingame, CA). Then, they were stained with KC57 anti-HIV capsid p24 antigen (CA p24) monoclonal antibody (mAb) (Coulter, Barcelona, Spain) and analyzed in an LSR II flow cytometer (BD Biosciences, Madrid, Spain). Cells were identified by morphological parameters. In addition, single-cell death was quantified by morphological parameters (forward versus side scatter plots). Quantification of HIV transfer was either assessed by the percentage of p24+ cells (using uninfected cells as a control) or by the mean fluorescence intensity.

**Time of Drug Addition Studies.** MT-4 cells were infected with NLA-3 virus at a multiplicity of infection of 0.5, and then they were incubated for 1 h at 20°C in the presence or absence of test compounds (AR177, AMD3100, ALX-40-4C, POL3026, C94, T-20, or AZT). Next, they were washed twice with ice-cold PBS, and compounds were added at various times after infection or cells were cultured in the absence of drug. The concentration of the different compounds was used high enough to block completely HIV replication (roughly 100-fold its EC50). Virus production as quantity of p24 antigen in supernatant was determined 30 h after infection (Armand-Ugón et al., 2005).

**Flow Cytometry Analysis.** Staining of chemokine receptor CXCR4 and CCR5, and the CD45 and CD4 receptor on CEM-CCR5 cell line was performed as reported previously (Pauls et al., 2007). In brief, 0.2 × 10⁶ cells were washed in PBS, and then they were incubated for 20 min at room temperature with mAbs anti-CD45 conjugated with fluorescein isothiocyanate, 12G5 (anti-CXCR4)-phycoerythrin, 2D7 (anti-CCR5)-allophycocyanin, and Leu3a (anti-CD4)-peridin chlorophyll protein (BD Biosciences, San Jose, CA), and with or without various drugs. The cells were then washed with 1× PBS and fixed in PBS containing 1% formaldehyde. Cells were analyzed by flow cytometry in an LSR II system (BD Biosciences). Data were acquired and analyzed with FACSDiva software (BD Biosciences). AMD3100, PRO140, Leu3a, and POL3026 were tested at different concentrations. The compound concentration required to inhibit mAb binding by 50% (IC50) was calculated. To evaluate whether differences in 12G5 mAb binding were due to CXCR4 downregulation, parallel experiments were done at 4°C (30 min of incubation) and 37°C (15 min of incubation). The IC50 of POL3026, AMD3100, and the chemokine SDF-1α was calculated for each condition.

**Measurement of Intracellular Calcium Concentrations.** The intracellular calcium concentrations [Ca²⁺]i were determined as described previously (Esté et al., 1999b). In brief, CEM-CCR5 cells or THP-1 cells were loaded with Fluo-4 at a 2.5 μM (Sigma-Aldrich). Fluorescence was measured in a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific, Helsinki, Finland). Cells (1 × 10⁶) were first stimulated with dilution buffer (control) or test compound at various concentrations. As a second stimulus, 100 ng/ml SDF-1α, RANTES, MIP-1α, or MIP-1β (all 1000 ng/ml) were used to induce [Ca²⁺]i signaling. The second stimulus was added 120 s after the first stimulus.

**Chemotaxis Assay.** The bottom chambers of HTS Transwell-96 chambers of 5-μm pore (Corning, Schiphol-Rijk, The Netherlands) were filled with 150 μl of RPMI 1640 medium containing 20 ng/ml of the chemoattractant SDF-1α and different concentrations of POL3026 or AMD3100, and the mixture was preincubated for 30 min at 37°C. Then, CEM-CCR5 cells (0.25 × 10⁶ in 50 μl of RPMI 1640 medium) were loaded onto the upper microchamber, and the assembled system was incubated for 3 h at 37°C, 5% CO2. After incubation, migrating cells were recovered from the lower chamber, and they were counted on a FACS-Calibur flow cytometer. Data are expressed as migration index (number of cells migrated in response to the chemoattractant plus the control, relative to the number of cells that migrated randomly to medium only).

**Coreceptor Switch Assay (Prolonged Culture of HIV-1 Strains in Sup-T1 Cells).** Sup-T1 cells (1.5 × 10⁶) were infected with 13 ng of p24 antigen from the HIV-1 R5 (nonsyncytium inducing) 168.1 virus. To obtain a 168.1 stock, 5 × 10⁶ Sup-T1 cells were transfected with 2 μg of proviral DNA using 0.4-cm cuvettes (Bio-Rad Laboratories, Hercules, CA) at 230 V and 950 μF.

Parallel cultures with different inhibitory conditions were maintained. The starting concentration of each compound was deter-
mined by its EC_{50} value in PBMC when tested against 168.1 virus. Twice a week, the cultures were passed by splitting one fifth in fresh media containing or not the specific inhibitor. Concentration for TAK-779 was adjusted along the passages to maintain a similar replication rate, and POL3026 was maintained at 1 μg/ml. Before each passage, each culture was controlled, and the detection of syncytia was scored. p24 in the supernatant of each culture was evaluated once a week with a commercial ELISA (Innotest HIV-Ag; Innogenetics). The R5, R5X4, or X4 phenotype was determined by evaluating the infectivity of the viruses in U87-CD4 and MT-2 cells.

When cultures were stopped, viral stocks were generated in Sup-T1 cells in the absence of compound. Viral stocks were aliquoted and stored at −80°C for future phenotype analysis. Cell pellets were used for the genetic analysis of proviral forms.

**Development of Resistant HIV-1.** MT-4 cells (0.1 × 10^6) were incubated with the HIV-1 NL4-3 or HXB2 virus in 48-well plates in a final 0.7-ml volume of growth medium. Passages were started with a POL3026 concentration of 0.0005 μg/ml (5-fold its EC_{50}). After 4, 5, or 6 days, depending on the cytopathic effects, supernatants were used to infect fresh MT-4 cells. The POL3026 concentration was progressively increased, finishing the passages when the concentration reached 0.034 and 0.043 μg/ml for virus A and virus B, respectively.

**Sequence Analysis.** Genomic DNA from infected cells was extracted using the QIAamp DNA blood mini kit (QIAGEN, Barcelona, Spain). Expand High Fidelity PCR System from Roche Diagnostics (Mannheim, Germany) and dNTP from Applied Biosystems (Madrid, Spain) were used for DNA template generation from the extracted DNA. Before sequencing, the amplified DNA was purified with the QIAquick PCR purification kit (QIAGEN). The env gene (5514–8910) was amplified with primers 5'-gataagacccattgctgt-3' and 5'-ttc-ctctggttagaatctg-3' as described previously (Armand-Ugón et al., 2005). Amplified DNA from the gp120 gene was sequenced with several primers (Armand-Ugón et al., 2005), with the ABI Prism BIGDYTer Terminator 3.1 kit (Applied Biosystems), and data were collected with the ABI Prism 3100 Avant Genetic Analyzer. Sequences were analyzed with the Sequencer 4.5 software (Gene Codes, Ann Arbor, MI) and edited with BioEdit software. Amino acid positions were numbered according to HXB2 (Los Alamos database).

**Growth Kinetics of Viruses.** Parallel cultures of MT-4 cells exposed to the same multiplicity of infection of virus (HXB2wt, HP41resA, HP38resB, and the AMD3100-resistant virus) were established. Infections were maintained during 5 days, and supernatant was collected each day for p24 quantification with a commercial ELISA (Innotest HIV-Ag; Innogenetics). Tricnate values from days 1, 2, 3, 4, and 5 were obtained.

**Growth Competition Assay.** Dual infection/competition experiments were performed with MT-4 cells on 24-well plates for 133 days. Uninfected cultures were used as negative control, whereas untreated infected cultures (wt HXB2, HP41resA, HP38resB, and the AMD3100-resistant virus) were established. Infections were maintained during 5 days, and supernatant was collected each day for p24 quantification with a commercial ELISA (Innotest HIV-Ag; Innogenetics). Tricnate values from days 1, 2, 3, 4, and 5 were obtained.

**Multiparametric Evaluation of HIV Envelope Function.** We have developed a simple method to evaluate the mode of action of HIV entry inhibitors through the evaluation of cell-to-cell interactions between HIV-infected and uninfected cells (Blanco et al., 2005). POL3026 efficiently blocked single-cell-death of CD4 T cells induced by MOLT-4/NL4-3 and MOLT4/C1-1-SI cells expressing X4 HIV-1 glycoproteins, but it did not prevent cell death induced by MOLT-4/BaL (R5) cells (data not shown). HIV transfer from infected to uninfected cells, as assessed by the percentage of p24+ cells (using uninfected cells as a control) could not be blocked by POL3026, AMD3100, the fusion inhibitor C34, and the RT inhibitor AZT (Fig. 2A). As shown previously (Blanco et al., 2005), only agents targeting the interaction of gp120 with CD4 (anti-CD4 antibody leu3A), blocked HIV cell-to-cell transfer, suggesting that POL3026 works at a step later than virus attachment to CD4.

**Results**

**Potent Anti-HIV Activity of POL3026 against a Broad Panel of HIV Strains.** We used a standard drug screening assay that is generally used in our laboratory for the through-put evaluation of candidate antiviral agents (Esté, 2003; Menéndez-Arias and Esté, 2004). As shown in Table 1, POL3026 proved to be highly potent against several X4 HIV strains. An EC_{50} value of 0.0001 μg/ml (0.05 nM) was calculated for the HIV-1 NL4-3 wild-type virus; thus, at least 10-fold more potent than the well-known CXCR4 antagonist AMD3100. POL3026 showed similar anti-HIV activity against viruses resistant to current antiviral agents, such as the RT inhibitors nevirapine and efavirenz or the fusion inhibitor T-20, and against the HIV-2 ROD strain or HIV-1 strains from different subtypes (A, B, D, F, and O) (Table 1). There was no evidence of synergy or antagonism when POL3026 was tested in combination with AZT, AMD3100, or T-20. Only additive effects were observed (data not shown).

POL3026 was not cytotoxic at any of the concentrations tested (up to 125 μg/ml). The 50% cytotoxic concentrations (CC_{50}) of all compounds tested are shown in Table 1.

**POL3026 Was Active against R5X4 and X4 Strains in PBMC.** Dualtropic (R5X4) HIV-1 strains preferentially use CXCR4 as entry coreceptor (Yi et al., 2005). POL3026 blocked the replication of R5X4 strains (three clinical isolates and the 89.6 HIV strain) with similar potency to that seen with HIV-1 strains of X4 phenotype (Table 1), with the exception of HIV-1 CI2 that was 6-fold less sensitive to POL3026 compared with the NL4-3 strain in PBMC. In addition, POL3026 seemed to be less effective against the NL4-3 strain in PBMC than in MT-4 cells. Although this difference may be a consequence of variation in the models used (e.g., different cell types, incubations times, and virus growth readouts), it may also reflect differences in coreceptor expression in stable and primary cells that affect the activity of the compound.

**Multiparametric Evaluation of HIV Envelope Function.** We have developed a simple method to evaluate the mode of action of HIV entry inhibitors through the evaluation of cell-to-cell interaction between HIV-infected and uninfected cells (Blanco et al., 2005). POL3026 efficiently blocked single-cell-death of CD4 T cells induced by MOLT-4/NL4-3 and MOLT4/C1-1-SI cells expressing X4 HIV-1 glycoproteins, but it did not prevent cell death induced by MOLT-4/BaL (R5) cells (data not shown). HIV transfer from infected to uninfected cells, as assessed by the percentage of p24+ cells (using uninfected cells as a control) could not be blocked by POL3026, AMD3100, the fusion inhibitor C34, and the RT inhibitor AZT (Fig. 2A). As shown previously (Blanco et al., 2005), only agents targeting the interaction of gp120 with CD4 (anti-CD4 antibody leu3A), blocked HIV cell-to-cell transfer, suggesting that POL3026 works at a step later than virus attachment to CD4.

**POL3026 Acted as a CXCR4 Antagonist in a Time of Addition Assay.** In time of drug addition experiments, a synchronized infection is established, and compounds are added at different times after infection. Virus production is measured after one cycle of replication. As shown in Fig. 2B, similar to the CXCR4 agents AMD3100 or Alelix-4C, POL3026 began to loose its activity if addition was delayed for 15 min, and the kinetics of virus growth was different from that seen for the binding inhibitor AR177, the gp41-dependent inhibitors C34 and T-20, or the RT inhibitor AZT, suggesting that POL3026 prevents infection by blockade of HIV coreceptors.
POL3026 Inhibited 12G5 mAb Staining and SDF-1α-Induced Intracellular Ca²⁺ Signaling and Chemotaxis.
To verify the specificity of POL3026 for CXCR4, its capacity to interfere with the staining of monoclonal antibodies against CXCR4, CCR5, CD45, or CD4 was tested. CEM-CCR5+ cells were stained with monoclonal antibodies alone or together with different compounds with known epitope specificities. POL3026 inhibited the staining of CXCR4+ cells with mAb 12G5 in a dose-dependent manner (IC₅₀ value of 0.0005 μg/ml) (Fig. 3B). Conversely, POL3026 did not interfere with the staining by mAbs specific for CCR5, CD45, or CD4 (data not shown). To determine whether the inhibition of the CXCR4 mAb staining was due to a down-regulation of CXCR4 or only to a masking of the epitope recognized by the 12G5 antibody, we calculated the IC₅₀ value for POL3026 (0.0067 ± 0.005 μg/ml) and AMD3100 (0.0061 ± 0.0022 μg/ml) at 37°C did not change significantly at 4°C (6- and 2-fold difference, respectively). Conversely, the IC₅₀ of the agonist chemokine SDF-1α (1.03 μg/ml) was 31-fold higher at 4°C, reflecting its capacity to down-regulate and mask the 12G5 mAb epitope. To further evaluate the interaction of POL3026 with CXCR4 coreceptor, we tested its effect on chemokine-induced [Ca²⁺] signaling. POL3026 by itself did not induce Ca²⁺ mobilization in CEM or THP-1 cells. POL3026 blocked Ca²⁺ signaling induced by the natural ligand of CXCR4 SDF-1α in both cell lines tested (Fig. 3C). The specificity of POL3026 was further demonstrated as it could not affect the Ca²⁺ mobilization induced by CCR5-specific chemokines RANTES, MIP-1α, and MIP-1β (data not shown), confirming the specificity of POL3026 for CXCR4. Furthermore, POL3026 showed a potent inhibition of the chemotactic response to SDF-1α by CXCR4+ cells (Fig. 3D). Taken together, these results suggest that POL3026 does not induce down-regulation, and it is a potent antagonist of CXCR4.

Anti-HIV Activity in Macrophages and Lymphoid Tissue Cultures. POL3026 blocked the replication of the macrophage-tropic X4 HIV-1 strain J130.3 in monocyte-derived macrophages. As shown in Fig. 4A, POL3026 at a concentration of 0.008 μg/ml inhibited virus replication by 90%. No activity was observed when tested against the R5 BaL strain (data not shown). None of the concentrations used were cytotoxic (data not shown).

Tissue culture studies may be an approximation to the in vivo complex environment. The potency of POL3026 was tested against HIV with R5 (BaL), R5X4 (89.6), and X4 (NL4-3) tropism in lymphoid tissue culture from tonsils ex vivo. As shown in Fig. 4B, POL3026 blocked the replication of X4 and R5X4 strains to the same extent as AZT and AMD3100. Conversely, no effect on R5 HIV-1 BaL replication was observed, whereas it could be blocked by the CCR5 antagonist TAK-779.

Antiviral Activity of POL3026 against NL4-3 Resistant to SDF-1α and AMD3100. As shown in Table 1, POL3026 was able to block the replication of the NL4-3 strains that were made resistant to AMD3100, albeit 200-fold loss in sensitivity. Likewise, the SDF-1α-resistant HIV-1

### Table 1

<table>
<thead>
<tr>
<th>HIV Strain</th>
<th>Subtype</th>
<th>Cells</th>
<th>EC₅₀</th>
<th>T-20</th>
<th>AZT</th>
<th>Nevirapine</th>
<th>Efavirenz</th>
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<tbody>
<tr>
<td>NL4-3 wt</td>
<td>B</td>
<td>MT-4</td>
<td>0.0001</td>
<td>0.001</td>
<td>0.07</td>
<td>0.004</td>
<td>0.2</td>
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<td>0.001</td>
<td>0.01</td>
<td>0.0005</td>
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<td>0.002</td>
<td>0.0002</td>
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<td>MT-4</td>
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<td>0.001</td>
<td>0.001</td>
<td>0.0007</td>
<td>&gt;2</td>
</tr>
<tr>
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<td>MT-4</td>
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<td>0.004</td>
<td>0.4</td>
<td>0.004</td>
<td>0.0001</td>
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<td>MT-4</td>
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<td>0.001</td>
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<td>0.2</td>
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<td>3</td>
<td>0.008</td>
<td>0.01</td>
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<td>SDFres</td>
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<td>0.008</td>
<td>0.01</td>
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<tr>
<td>92BR014</td>
<td>B</td>
<td>MT-4</td>
<td>0.00001</td>
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<td>0.0003</td>
<td>0.007</td>
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<td>0.0001</td>
<td>0.007</td>
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<tr>
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<td>0.0003</td>
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<td>0.008</td>
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<td>0.004</td>
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<td>MT-4</td>
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<td>0.0002</td>
<td>0.00004</td>
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<tr>
<td>ROD (HIV-2)</td>
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<td>MT-4</td>
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<td>0.001</td>
<td>0.005</td>
<td>&gt;2</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>NL4-3 wt</td>
<td>B</td>
<td>PBMC</td>
<td>0.005</td>
<td>0.006</td>
<td>&gt;0.2</td>
<td>0.2</td>
<td>0.001</td>
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<tr>
<td>BaL</td>
<td>B</td>
<td>PBMC</td>
<td>&gt;0.1</td>
<td>&gt;0.5</td>
<td>0.006</td>
<td>0.003</td>
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<td>89.6</td>
<td>B</td>
<td>PBMC</td>
<td>0.007</td>
<td>0.008</td>
<td>&gt;0.2</td>
<td>0.2</td>
<td>0.0006</td>
</tr>
<tr>
<td>CI2</td>
<td>B</td>
<td>PBMC</td>
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<td>0.05</td>
<td>&gt;0.2</td>
<td>0.2</td>
<td>0.001</td>
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<tr>
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<td>B</td>
<td>PBMC</td>
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<td>0.04</td>
<td>&gt;0.2</td>
<td>0.2</td>
<td>0.001</td>
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<tr>
<td>CI4</td>
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<td>PBMC</td>
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<td>0.05</td>
<td>&gt;0.2</td>
<td>0.2</td>
<td>0.0003</td>
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<tr>
<td>HXB2 wt</td>
<td>B</td>
<td>MT-4</td>
<td>0.00004</td>
<td>0.003</td>
<td>0.001</td>
<td>0.04</td>
<td></td>
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<tr>
<td>HXB2.41</td>
<td>B</td>
<td>MT-4</td>
<td>0.00004 (1)</td>
<td>0.001 (1)</td>
<td>0.001 (1)</td>
<td>0.03 (1)</td>
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<tr>
<td>HP41resA</td>
<td>B</td>
<td>MT-4</td>
<td>0.0005 (12)</td>
<td>0.03 (10)</td>
<td>0.001 (1)</td>
<td>0.03 (1)</td>
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</tr>
<tr>
<td>HP38resB</td>
<td>B</td>
<td>MT-4</td>
<td>0.001 (25)</td>
<td>0.07 (23)</td>
<td>0.001 (1)</td>
<td>0.08 (2)</td>
<td></td>
</tr>
</tbody>
</table>

| CC₅₀ | >125 | >5 | >1 | >0.2 | >5 | >5 | >1 | >2 | >0.1 |

S.D. for control HIV were cytotoxic (data not shown).
strain was also cross-resistant to POL3026 and AMD3100 (6- and 8-fold increase in EC50, respectively). Compounds acting at the reverse transcriptase level (AZT) or at other entry step (C34 or T-20) were equally active against all strains tested. Thus, amino acid changes conferring resistance to AMD3100 and SDF-1α in the NL4-3 backbone affect the sensitivity to POL3026, suggestive of a similar mode of action.

**POL3026 Prevented the Emergence of X4 Viruses from the R5 168.1 Strain.** We have standardized an in vitro model that allows us to study coreceptor switch of an R5 virus to X4 or R5/X4 in cell culture (Moncunill et al., 2008). The model is based on the prolonged culture of viruses in the lymphoid cell line Sup-T1 that express low levels of CCR5, allowing R5 viruses to replicate at a low rate. After few passages, the R5 HIV-1 168.1 expanded its use to CXCR4 followed by syncytium formation and a peak in p24 viral antigen detection (Fig. 5A). In the presence of a CCR5 antagonist, TAK-779, coreceptor switch could be delayed, probably due to the lower replicating rate compared with the control culture in the absence drug pressure. However, after 17 more passages, HIV-1 168.1 gained resistance to the CCR5 antagonist through coreceptor switch (i.e., increased virus replication). Conversely, in the presence of POL3026, the emergence of CXCR4-using viruses was prevented. The change of phenotype of virus recovered from TAK-779 culture and untreated cells was confirmed by virus growth in CXCR4+ MT-2 and U87 CD4+ CXCR4+ cells, and it was concomitant to the emergence of mutations in the V3 loop region of gp120 (data not shown).

**POL3026-Resistant Viruses.** MT-4 cells were infected with HIV-1 HXB2 in the absence (HXB2.41) or presence of...
increasing concentrations of POL3026 for up to 205 days (41 passages in cell culture; Fig. 5B). Two virus isolates (HP41resA and HP38resB) were recovered and titered for anti-HIV evaluation. The parental HIV-1 HXB2-wt and the strain passed without POL3026 HXB2.41 were equally inhibited by all anti-HIV compounds tested. Virus strains grown in the presence of POL3026 (HP41resA and HP38resB) were 12- and 25-fold resistant to POL3026 and 10- and 23-fold cross-resistant to AMD3100, respectively, but they remained equally sensitive to AZT and nevirapine (Table 1). The analysis of the amino acid sequence of gp120 derived from proviral DNA from HIV-infected cells revealed the presence of mutations as a result of selective pressure with POL3026. Mutations were detected mainly in the V3 loop of gp120, which is thought to interact with the HIV-1 coreceptors. HP41resA and HP38resB had one mutation in common, N325D, in the V3 loop region that contributes to the acidification of the V3. Each virus strain contained two other mutations in the V3 loop region (Fig. 5C).

Drug resistance may affect the replication capacity of HIV-1 in the absence of drug. To evaluate the fitness cost of mutations conferring resistance to POL3026, growth competition experiments between HP41resA or HP38resB and the wild-type HXB2.41 strain were performed. However, after 133 days in cell culture, there was no clear indication of a better-fit virus as measured by quantification of the proviral DNA sequence corresponding to POL3026-resistant or wild-type virus, suggesting little differences in virus fitness. In single infection assays, we compared the growth kinetics of HP41resA and HP38resB with that of the HXB2.41 and the AMD3100-resistant virus shown to have reduced fitness (Armand-Ugón et al., 2003b) (Fig. 5D). The growth of the HP41resA strain seemed to be similar to that of the wild type. Conversely, the replication of HIV-1 HP38resB was similar to that of the AMD3100-resistant virus, suggesting that an increase in drug resistance to POL3026 may lead to impaired replication capacity.

**Discussion**

Previous work selected POL3026 by its plasma stability, high selectivity for CXCR4, and favorable pharmacokinetic properties in dogs (DeMarco et al., 2006). Here, we characterize the mode of action of POL3026 as an anti-HIV agent. We confirmed that POL3026 binds to CXCR4 and interferes with the binding of the mAb directed against this chemokine receptor. Moreover, POL3026 did not induce an intracellular Ca\(^{2+}\) flux, but it interfered with the calcium flux induced by SDF-1α, suggesting that POL3026 acts as an antagonist of CXCR4. Furthermore, multivariate parametric evaluation of HIV envelope function and time of addition experiments suggests that POL3026 blocks HIV replication at a time or site that corresponds with the interaction with the virus coreceptor.

POL3026 was active against X4 and R5X4 HIV strains, including clinical isolates and virus strains that are resistant to other drug classes but lost activity against HIV-1 strains that had the same genetic background (i.e., NL4-3) and had been made resistant to other ligands of CXCR4. Cross-resistance may not be obligatory, because ligands such as AMD3100, SDF-1α, and POL3026 may interact differently with CXCR4, and they may be "seen" differently by HIV-1 strains with distinct HIV envelopes. However, when comparing three virus isolates with a similar genetic backbone (i.e., NL4-3) cross-resistance suggests a similar mode of action.

POL3026 inhibited the replication of a broad panel of HIV strains, including HIV-2 ROD and different HIV-1 subtypes (A, B, D, F, and O). Moreover, POL3026 was highly potent against a panel of drug-resistant viruses, including the RT inhibitors AZT, nevirapine, or efavirenz, and the fusion inhibitors T-20. The EC\(_{50}\) value of POL3026 for all these viruses was in the nanomolar and subnanomolar range, making POL3026 the most active anti-CXCR4 agent known to date. POL3026 was also effective in a nanomolar range of concentrations against dualtropic strains (89.6 and three clinical HIV-1 isolates) when tested in PBMC, or in lymphoid tissue cultures. Its anti-HIV activity against dualtropic viruses could be expected because R5X4 strains may preferentially use CXCR4 (Yi et al., 2005). However, some of the clinical isolates showed a loss of sensitivity to POL3026 and AMD3100 compared with the NL4-3 strain in PBMC, probably because of their capacity to use both coreceptors. POL3026 did not inhibit the replication of R5 tropic virus, but it blocked the replication of macrophage tropic HIV-1

![Fig. 4](https://example.com/fig4.png)
J130.3 that uses CXCR4. POL3026 showed potent anti-HIV activity in primary cells and in lymphoid tissue culture ex vivo, confirming its potential as a selective agent against HIV strains that use CXCR4.

From the above-mentioned results, it was not surprising the emergence and location of mutations developed under selective pressure with this compound. These mutations occur mainly in the V3 loop of gp120, and four mutations...
with excellent pharmacokinetics and the potential to become 
CXCR4 drug combinations may be used to prevent the emer-
results further support the hypothesis that CCR5 and 
from patients to switch coreceptor preference. Therefore, our 
2D7, a CCR5 monoclonal antibody) than with zidovudine 
culture experiments, three of six virus strains with the in-
ition of HIV-1 infection until later stages of disease, but it may 
individuals may harbor X4 viruses (Brumme et al., 2005; 
X4 viruses are not favorably selected during the natural evolu-
ion of HIV-1 infection until later stages of disease, but it may 
may coexist as a minor subpopulation that goes unnoticed by 
available methods of detection (Weber et al., 2006). In cell 
culture experiments, three of six virus strains with the 
intrinsic capacity to expand their coreceptor use switched to 
CXCR4 faster with CCR5 drug pressure (TAK-779, CCL5, or 
CCL5 monoclonal antibody) than with zidovudine 
(Moncunill et al., 2008). In clinical trials of patients treated 
with a CCR5 agent, more patients had a change in tropism to 
duotropic/mix (R5/X4) or X4 at time of failure (Nelson et al., 
2007), drawing attention to the propensity of virus isolates 
from patients to switch coreceptor preference. Therefore, our 
results further support the hypothesis that CCR5 and 
CXCR4 drug combinations may be used to prevent the emerg-
ence of CXCR4-using viruses or the selection of minor X4 
populations already present that may go undetected. The 
opimization of POL3026 may lead to prototype compounds with 
excellent pharmacokinetics and the potential to become 
candidates for clinical evaluation.

Acknowledgments

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Lalezari J, Goodrich J, DeJesus E, Lampiris H, Gulick R, Saag M, Ridgway C, 


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