

Anti-HIV Activity and Resistance Profile of the CXC Chemokine Receptor 4 Antagonist POL3026^[S]

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ABSTRACT

We have studied the mechanism of action of Arg^{*}-Arg-Nal²-Cys(1×)-Tyr-Gln-Lys-(D-Pro)-Pro-Tyr-Arg-Cit-Cys(1×)-Arg-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-1 α -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-

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ABBREVIATIONS: HIV, human immunodeficiency virus; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; FCS, fetal calf serum; SI, syncytium-inducing; PBMC, peripheral blood mononuclear cells; SDF-1, stromal cell-derived factor-1; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; RT, reverse transcriptase; AZT, 3-Azido-3-deoxythymidine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; wt, wild type; MDM, monocyte-derived macrophages; mAb, monoclonal antibody; POL3026, Arg^{*}-Arg-Nal²-Cys(1×)-Tyr-Gln-Lys-(D-Pro)-Pro-Tyr-Arg-Cit-Cys(1×)-Arg-Gly-(D-Pro)^{*}; AMD3100, 1-[[4-(1,4,8,11-tetrazacyclotetradec-1-ylmethyl)phenyl]methyl]-1,4,8,11-tetrazacyclotetradecane; ALX-40-4-C, Ac-(D-Arg)9-NH₂; TAK-779, dimethyl-[[4-[[3-(4-methylphenyl)8,9-dihydro-7H-benzo[7]annulene-6-carbonyl]amino]-phenyl]methyl]-(oxan-4-yl)azanium chloride.

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 temporarily separated, have been developed, particularly the chemokine coreceptors 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, providing 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 the β -hairpin peptide 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 having various amino acid combinations in the linker region. After several rounds of optimization, POL3026 was obtained. Here, we show that POL3026 may potently block X4 HIV-1 replication and prevent the emergence of CXCR4-using HIV-1.

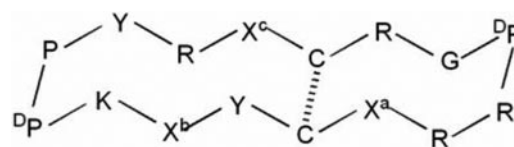
Materials and Methods

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 μ g/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 of buffy coats obtained from the Catalonia Banc de Sang i Teixits (Barcelona, Spain). PBMC from each donor were mixed equally and resuspended at 50×10^6 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.

Compounds. The synthesis, purification, and chemical characterization of POL3026 (Fig. 1) were performed as described previously (DeMarco et al., 2006). The chemokine SDF-1 α (natural ligand of CXCR4) and the natural ligands of CCR5 MIP-1 α , MIP-1 β , 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 (Doranz 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 NL4-3 strain that is resistant to T-20/C34 has been described previously (Armand-Ugón et al., 2003a; Menéndez-Arias and Esté, 2004). The IRL98 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-1 α -resistant strain were derived after sequential passage of the NL4-3 virus in the presence of increasing concentrations of AMD3100 or SDF-1 α in MT-4 cells (de Vreese et al., 1996). The X4 HIV-1 clinical isolate CI1, and the dualtropic CI2, CI3, and CI4 were



POL3026: X^a=2-Nal; X^b=Q; X^c=Cit

Fig. 1. Structure of POL3026.

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 (EC_{50}) were calculated as valid when the variation between replicates was less than 4-fold. Mean $EC_{50} \pm$ S.D. for control compounds AZT and AMD3100 was 0.0008 ± 0.0002 and 0.001 ± 0.0004 μ g/ml, respectively. Cut-off value in which a virus was considered resistant was greater than 4-fold increase of the EC_{50} 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/ 10^6 cells) or mock-infected for 3 h at 37°C. Thereafter, they were washed twice with $1 \times$ phosphate-buffered saline (PBS). Infected cells were seeded in 96-well plates (0.15×10^6 cells/well), and they were incubated 7 days at 37°C, 5% CO_2 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, $1 \times$ 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 PBMC were performed three times. To ensure the reproducibility of the assay, the mean EC_{50} and S.D. values were calculated for the control drugs AZT and AMD3100 against the NL4-3 wt strain. The EC_{50} was 0.001 ± 0.0005 μ g/ml ($n = 3$) and 0.006 ± 0.001 μ g/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 μ l of culture supernatant was replaced by 20 μ l 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 $1 \times$ PBS, dissected into 2- to 3-mm blocks, and placed on top of gelatin sponge gels (Espongostan; Prifar, Porto, Portugal) with RPMI 1640 medium, 10% FCS, and penicillin/streptomycin. HIV-1 infection was carried out with 1.5 μ l 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 (2×10^5) were cocultured with 2×10^5 MOLT-4/CCR5 cells chronically infected with the X4 isolates NL4-3 and CI-1-SI. After 24 h of coculture, cells were washed with $1 \times$ 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 NL4-3 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, C34, 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 used was high enough to block completely HIV replication (roughly 100-fold its EC_{50}). 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, 0.2×10^6 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 $1 \times$ 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% (IC_{50}) was calculated. To evaluate whether differences in 12G5 mAb binding were due to CXCR4 down-regulation, parallel experiments were done at 4°C (30 min of incubation) and 37°C (15 min of incubation). The IC_{50} of POL3026, AMD3100, and the chemokine SDF-1 α was calculated for each condition.

Measurement of Intracellular Calcium Concentrations. The intracellular calcium concentrations [Ca^{2+}] 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 μ M (Sigma-Aldrich). Fluorescence was measured in a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific, Helsinki, Finland). Cells (1×10^6) were first stimulated with dilution buffer (control) or test compound at various concentrations. As a second stimulus, 100 ng/ml SDF-1 α , RANTES, MIP-1 α , or MIP-1 β (all 1000 ng/ml) were used to induce [Ca^{2+}] signaling. The second stimulus was added 120 s after the first stimulus.

Chemotaxis Assay. The bottom chambers of HTS Transwell-96 chambers of 5- μ m pore (Corning, Schiphol-Rijk, The Netherlands) were filled with 150 μ l of RPMI 1640 medium containing 20 ng/ml of the chemoattractant SDF-1 α and different concentrations of POL3026 or AMD3100, and the mixture was preincubated for 30 min at 37°C. Then, CEM-CCR5 cells (0.25×10^6 in 50 μ l of RPMI 1640 medium) were loaded onto the upper microchamber, and the assembled system was incubated for 3 h at 37°C, 5% CO_2 . After incubation, migrating cells were recovered from the lower chamber, and they were counted on a FACSCalibur flow cytometer. Data are expressed as migration index (number of cells migrated in response to the chemoattractant plus the compound, 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 (1.5×10^5) were infected with 13 ng of p24 antigen from the HIV-1 R5 (nonsyncytium inducing) 168.1 virus. To obtain a 168.1 stock, 5×10^6 Sup-T1 cells were transfected with 2 μ g of proviral DNA using 0.4-cm cuvettes (Bio-Rad Laboratories, Hercules, CA) at 250 V and 950 μ F.

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 $\mu\text{g/ml}$. 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 $\mu\text{g/ml}$ (5-fold its EC_{50}). After 4, 5, or 6 days, depending on the cytopathic effects, supernatants were used to infect new fresh MT-4 cells. The POL3026 concentration was progressively increased, finishing the passages when the concentration reached 0.034 and 0.043 $\mu\text{g/ml}$ 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'-gataaagccaccttgctagt-3' and 5'-ttc-taggtctcgagatactg-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 BIGDYE Terminator 3.1 kit (Applied Biosystems), and data were collected with the ABI Prism 3100 Avant Genetic Analyzer. Sequences were analyzed with the Sequencher 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). Triplicate 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 HXB2.41, HP41resA, and HP38resB) at a multiplicity of infection of 0.003 corresponded to positive controls (100% virus). The competition assay involved three separate dual infections with each resistant virus (HP41resA and HP38resB) plus the wt virus at different multiplicities of infection expressed by proportions (90% resistant virus plus 10% wt, 50% resistant plus 50% wt, and 10% resistant plus 90% wt). Every 5 to 7 days, the supernatant was used to infect fresh MT-4 cells, and aliquots of cells were harvested and stored at -80°C for subsequent analysis. Detection of each virus population was assessed by sequencing the V3 loop of gp120 as explained above.

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; Méndez-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 $\mu\text{g/ml}$ (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 $\mu\text{g/ml}$). 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/CI-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 lose 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-1 α -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 μ g/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 values of POL3026, AMD3100, and SDF-1 α at 37°C (both down-regulation and epitope masking may occur) and at 4°C (only masking of the epitope is evaluated). The IC₅₀ value for POL3026 (0.0067 \pm 0.005 μ g/ml) and AMD3100 (0.0061 \pm 0.0022 μ g/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-1 α (1.03 μ g/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⁺]_i 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-1 α in both cell lines tested (Fig. 3C). The specificity of POL3026 was further demonstrated as it could not affect the Ca²⁺ mobi-

lization induced by CCR5-specific chemokines RANTES, MIP-1 α , and MIP-1 β (data not shown), confirming the specificity of POL3026 for CXCR4. Furthermore, POL3026 showed a potent inhibition of the chemotactic response to SDF-1 α 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 μ g/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-1 α 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-1 α -resistant HIV-1

TABLE 1
Anti-HIV activity of POL3026

EC₅₀ is the concentration needed to inhibit by 50% the replication of HIV strain as measured by p24 production (when tested in PBMC) or for the inhibition of 50% of cell death as measured by the MTT assay (when tested in MT-4 cells). Results shown are the mean of three separate experiments performed in triplicate. S.D. for control compounds AZT and AMD3100 was 0.0008 \pm 0.0002 and 0.001 \pm 0.0004 μ g/ml, respectively. The S.D. for all values is shown in the Supplemental Table. Values in parentheses are -fold resistance, the relative loss of activity of the corresponding virus relative to the HXB2 wt strain calculated as the mean EC₅₀ of the virus strain/mean EC₅₀ of the HXB2 wt strain. CC₅₀ is the concentration needed to induce 50% cell death in mock-infected MT-4 cells as measured by the MTT assay.

HIV Strain	Subtype	Cells	EC ₅₀								
			POL3026	AMD3100	SDF-1 α	TAK-779	C34	T-20	AZT	Nevirapine	Efavirenz
<i>$\mu\text{g/ml}$</i>											
NL4-3 wt	B	MT-4	0.0001	0.001	0.07		0.004	0.2	0.0008	0.004	0.0004
K103N	B	MT-4	0.0001	0.001					0.0005	0.03	0.001
IRLL98	B	MT-4	0.00001	0.001					0.0006	>2	>0.1
Y181C	B	MT-4	0.0001	0.002					0.0002	0.9	0.002
Y188L	B	MT-4	0.00005	0.001					0.0007	>2	>0.1
CI-1-SI	B	MT-4	0.0002	0.004					0.4	0.004	0.0001
T20r/C34r	B	MT-4	0.0004	0.001	0.1		0.2	1	0.0005		
AMD3100res	B	MT-4	0.02	0.08	3		0.008	0.01	0.0005		
SDFres	B	MT-4	0.0006	0.008	2,2		0.008	0.01	0.0006		
92BR014	B	MT-4	0.00001	0.004	>1		0.00003	0.007	0.001	0.01	
UG92029	A	MT-4	0.0001	0.008	>1		0.0001	0.007	0.001	0.05	
39RW024	A	MT-4	0.00002	0.001	>1		0.0006	0.0003	0.001	0.04	
92UG021	D	MT-4	0.0001	0.002	>1		0.005	0.002	0.001	0.08	
92UG024	D	MT-4	0.0002	0.008	>1		0.002	0.003	0.0009	0.1	
93BR020	F	MT-4	0.00003	0.004	>1		0.01	0.004	0.002	0.09	
BCF06	O	MT-4	0.00008	0.004	>1		0.0002	0.00004	0.0005	0.09	
ROD (HIV-2)		MT-4	0.0001	0.001					0.0005	>2	>0.1
NL4-3 wt	B	PBMC	0.005	0.006		>0.2		0.2	0.001		
BaL	B	PBMC	>0.1	>0.5		0.006		0.003	0.0003		
89.6	B	PBMC	0.007	0.008		>0.2		0.3	0.0006		
CI2	B	PBMC	0.03	0.05		>0.2		0.2	0.001		
CI3	B	PBMC	0.002	0.04		>0.2		0.2	0.001		
CI4	B	PBMC	0.0005	0.05		>0.2		0.2	0.00003		
HXB2 wt	B	MT-4	0.00004	0.003					0.001	0.04	
HXB2.41	B	MT-4	0.00004 (1)	0.004 (1)					0.001 (1)	0.03 (1)	
HP41resA	B	MT-4	0.0005 (12)	0.03 (10)					0.001 (1)	0.03 (1)	
HP38resB	B	MT-4	0.001 (25)	0.07 (23)					0.001 (1)	0.08 (2)	
CC ₅₀			>125	>5	>1	>0.2	>5	>5	>1	>2	>0.1

strain was also cross-resistant to POL3026 and AMD3100 (6- and 8-fold increase in EC_{50} , 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-1 α 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

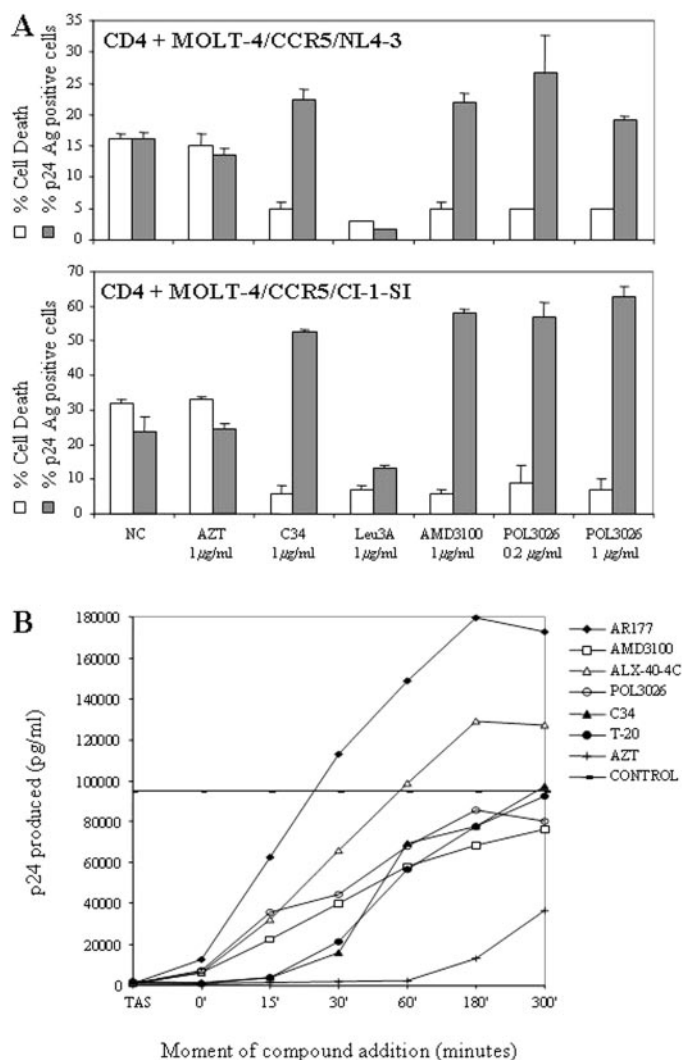


Fig. 2. A, effect of compounds (RT inhibitor AZT, fusion inhibitor C34, anti-CD4 mAb Leu3A, and CXCR4 antagonists AMD3100 and POL3026) on single cell death and p24 transfer to CD4 T cells, induced by coculture with two different sets of HIV-1 NL4-3 (X4) chronically infected MOLT-4/CCR5 cells. Single cell death is represented respect to control cocultures with uninfected MOLT-4/CCR5 cells. Results from two experiments performed in triplicates are represented. Error bars indicate S.D. B, effect of the addition of compounds (binding inhibitor AR177; CXCR4 antagonists AMD3100 and ALX-40-4C; POL3026; fusion inhibitors C34 and T-20; and RT inhibitor AZT) at different moments after initiation of infection on the p24 production by NL4-3 in MT-4 cells 30 h after infection. The compounds were used at a blocking concentration of HIV replication. TAS, temperature-arrested state. A representative time of addition experiment is shown. Similar results were obtained in four separate experiments.

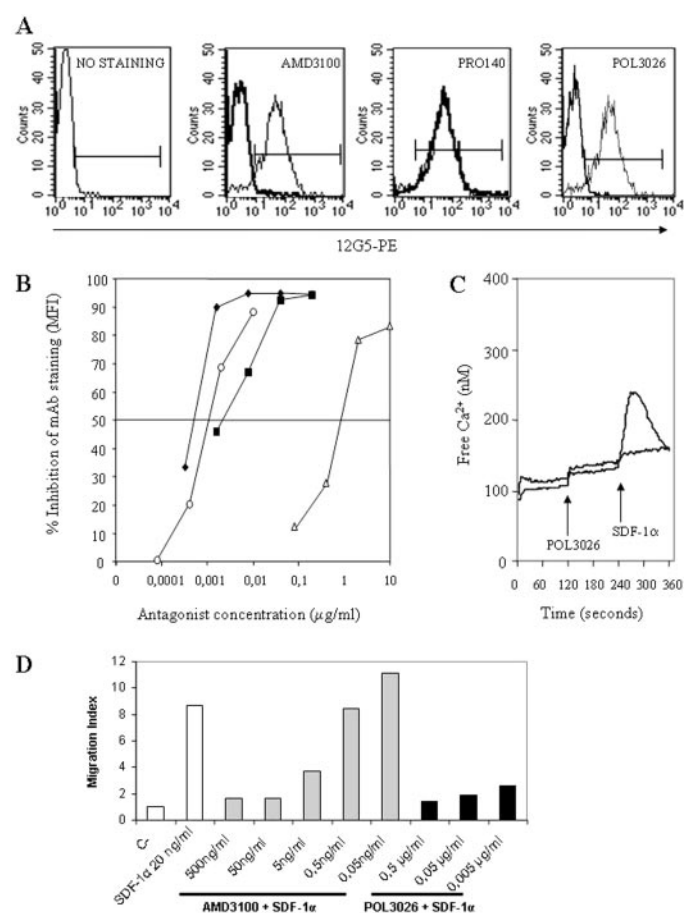


Fig. 3. A, inhibition of mAb 12G5 (anti-CXCR4) staining on CEM-CCR5 cells by different compounds (CXCR4 antagonist AMD3100 at 0.2 μ g/ml, anti-CCR5 mAb PRO140 at 10 μ g/ml, and POL3026 at 0.04 μ g/ml). B, dose-response curve of the inhibition of staining of mAb anti-CXCR4 12G5 by POL3026 (\blacklozenge) and the CXCR4 antagonist AMD3100 (\blacksquare), mAb anti-CCR5 2D7 by the anti-CCR5 mAb PRO140 (\triangle), and mAb anti-CD4 Leu3A by unstained Leu3A (\circ). C, calcium mobilization induced by 100 ng/ml SDF-1 α in 0.2×10^6 CEM-CCR5 cells is blocked by 1 μ g/ml POL3026. Representative experiments are presented in A to C. The results were confirmed in three separate experiments. D, CEM-CCR5 cells induced to migrate through a 5- μ m pore membrane by the CXCR4 ligand SDF-1 α (20 ng/ml) in the presence or absence of POL3026 or the CXCR4 antagonist AMD3100 as a control. Cell migration was quantified by flow cytometry, and results are expressed as migration index. Data shown is representative of experiments performed at least twice.

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 staining 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-1 α , suggesting that POL3026 acts as an antagonist of CXCR4. Furthermore, multiparametric 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-1 α , 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 inhibitor 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

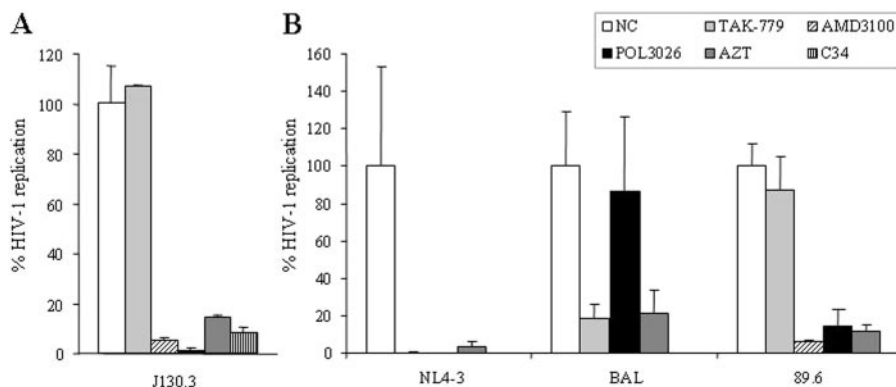
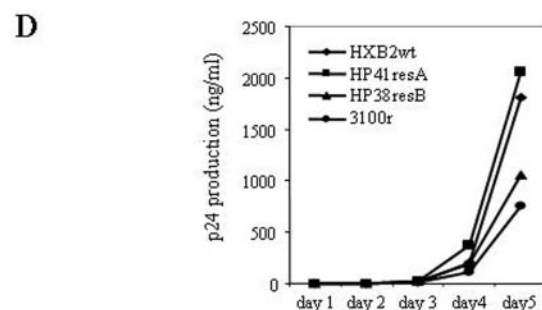


Fig. 4. A, inhibition of HIV-1 replication of J130.3 X4 strain in MDM. Concentrations of compounds used were TAK-779 (CCR5 antagonist), 1 $\mu\text{g}/\text{ml}$; AMD3100 (CXCR4 antagonist), 5 $\mu\text{g}/\text{ml}$; POL3026, 0.008 $\mu\text{g}/\text{ml}$; AZT (RT inhibitor), 0.2 $\mu\text{g}/\text{ml}$; and C34 (fusion inhibitor), 2 $\mu\text{g}/\text{ml}$. Graphic data are the mean of two experiments performed in triplicates. B, inhibition of replication of X4 HIV-1 NL4-3, R5 HIV-1 BAL, or R5X4 HIV-1 89.6 strains in tonsillar lymphocyte cultures. When tested, the represented concentrations of compounds were TAK-779, 1 $\mu\text{g}/\text{ml}$; AMD3100, 2 $\mu\text{g}/\text{ml}$; POL3026, 1 $\mu\text{g}/\text{ml}$; and AZT, 1 $\mu\text{g}/\text{ml}$. A representative experiment performed in triplicates is shown. Similar results were obtained in three separate experiments (tonsil tissues coming from different donors). A and B, values of p24 production at each point are represented relative to the p24 produced by the control culture without compound. Error bars indicate S.D. NC, no compound.

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



E

Virus strain	V3 Amino Acids																			
HXB2	C	T	R	P	N	-	-	-	-	N	N	T	R	K	R	I	R	I	Q	R
HP41 ^{resA}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-
HP38 ^{resB}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-
NL4-3	C	T	R	P	N	-	-	-	-	N	N	T	R	K	S	I	R	I	Q	R
AMD3100r	-	-	-	-	N,Y	-	-	-	-	S	-	T	R	-	-	-	-	-	V	-
SDFr	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	V	-
T134r	-	-	-	-	-	K	-	-	-	-	-	-	-	T	K	-	-	-	V	L

Fig. 5. A, growth of R5 HIV-1 168.1 strain in sustained infection of Sup-T1 cells and effect of the CCR5 antagonist TAK-779 or POL3026 on the evolution of its phenotype. Virus replication was measured as p24 antigen in the supernatant of cell culture. Low virus replication was reminiscent of R5 phenotype. Peak of p24 production reflected the gain of CXCR4 use. Representation of one of three separate experiments. B, development of resistance to POL3026. Two HXB2 resistant strains were obtained after passages with increasing concentrations of POL3026. The rate of POL3026 concentration increase for each resistant virus during the passages is represented. C, mutations in gp120 that confer resistance to POL3026. Residues are numbered according to the HXB2 gp120 sequence. D, growth kinetics of HXB2.41, HP41resA, HP3resB, and AMD3100-resistant virus. Virus replication was measured as p24 antigen in the supernatant of MT-4 cultures. Similar results were obtained in four separate experiments performed in triplicates. E, comparison of V3 loop mutations that emerged under different CXCR4 targeting compounds: POL3026, AMD3100 (de Vreese et al., 1996), the chemokine SDF-1 α (Schols et al., 1998), and the T134 CXCR4 inhibitor (Kanbara et al., 2001).

(Q310H, I320T, N325D, and A329T) are shared by viruses resistant to SDF-1 α (Schols et al., 1998), AMD3100 (de Vreese et al., 1996), and T134 (Kanbara et al., 2001) (Fig. 5E). The mutations did not have a clear fitness cost as measured by virus competition assays. However, growth kinetics indicates that HP38resB that is 25-fold resistant to POL3026 may have an impaired replication capacity. These results contrast with the marked reduced fitness of the AMD3100-resistant virus (Armand-Ugón et al., 2003b). However, the number of mutations (up to 11 mutations after selection of resistance) and the degree of resistance (up to 100-fold) could explain the fitness differences between POL3026 and AMD3100-resistant strains.

We also show that POL3026 prevents the emergence of CXCR4-using strains under conditions that are restricting for CCR5, a result that may have an important implication in the treatment of HIV+ individuals. Early work showing the reversion of X4 to R5 phenotype by a CXCR4 antagonist led us to suggest that virus coreceptor switch could be induced by selective drug pressure (Esté et al., 1999a), and recent studies have shown that roughly 20% of drug-naïve (untreated) individuals may harbor X4 viruses (Brumme et al., 2005; Moyle et al., 2005), a percentage that increases up to 58% among drug-experienced individuals (Melby et al., 2006). X4 viruses are not favorably selected during the natural evolution of HIV-1 infection until later stages of disease, but it 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 2D7, a CCR5 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 dualtropic/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 emergence of CXCR4-using viruses or the selection of minor X4 populations already present that may go undetected. The optimization of POL3026 may lead to prototype compounds with excellent pharmacokinetics and the potential to become candidates for clinical evaluation.

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