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Anti-HIV Activity and Resistance Profile of the CXCR4 Antagonist POL3026

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Running title: Anti-HIV activity of POL3026

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Abreviations: CXCR4, CXC chemokine receptor 4; CCR5, CC chemokine receptor 5; CXCL12, CXC chemokine ligand 12; SDF-1, stromal cell-derived factor-1; RANTES, regulated on activation normal T cell expressed and secreted; MIP-1α, macrophage inflamatory protein-1α; MIP-1ß, macrophage inflamatory protein-1ß; M-CSF, macrophage colony stimulating factor; EC_{50}, effective concentration 50 or the concentration needed to inhibit by a 50% the replication of HIV strain; CC_{50}, cytotoxic concentration 50 or the concentration needed to induce 50% cell death in mock-infected cells; IC_{50}, 50% inhibitory concentration or concentration needed to inhibit mAb binding

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by a 50%; mAb, monoclonal antibody; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; PBS, phosphate-buffered saline; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PBMC, peripheral blood mononuclear cells; AZT, 3-Azido-3-deoxythymidine; MTT, 3-[4,5-Dimetiltiazol-2-il]-2,5-difeniltetraxolium-bromide; P/S, penicillin streptomycin; SI, syncytium-inducing; NSI, non-syncytium-inducing; MOI, multiplicity of infection; $TCID_{50,}$ 50% tissue culture infectious doses; FITC, fluorescein isothiockyanate; PerCP, peridin chlorophyll protein.

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ABSTRACT

We have studied the mechanism of action of POL3026, a novel specific ß-hairpin mimetic CXCR4 antagonist. POL3026 specifically blocked the binding of anti-CXCR4 monoclonal antibody 12G5 and the intracellular Ca^{2+} signal induced by CXCL12. POL3026 consistently blocked the replication of HIV, including a wide panel of X4 and dualtropic strains and subtypes in several culture models, with 50% effective concentrations (EC_{50}) at the sub-nanomolar range making POL3026 the most potent CXCR4 antagonist described to date. However, AMD3100-resistant and SDF-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, namely 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 a R5 HIV-1 strain that may appear in the presence of anti-HIV agents targeting CCR5.

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INTRODUCTION

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 (Menendez-Arias and Este, 2004). Over the course of the infection, the coreceptor usage 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 cannot eradicate the virus from infected individuals and 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 (Este, 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-experience patients and others are in advanced clinical trials (Este 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 utilize CXCR4 (Westby et al., 2006).

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Bicyclams are the first class of CXCR4 agents described to block HIV replication (De Clercq, 2000; De Clercq, 2003; Este et al., 1999a) 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 was suspended after liver histology changes and liver and retinal toxicity were observed in animal research studies. However, a greater than one log reduction of X4 was observed in 4 out of 9 patients, and 3 out of the 4 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.

Recently, 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, with a MW of 2114, was designed starting from a truncated analogue 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.

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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 (MRC) 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 NIH AIDS Research and Reference Reagent Program. MT-4, THP-1, Sup-T1 and MT-2 cells were grown in RPMI 1640 and U87 cells were grown in DMEM (Invitrogen, Barcelona, Spain), supplemented with 10% fetal calf serum (FCS, Cambrex, Barcelona, Spain) and antibiotics, 2 U/ml penicillin and 2 μg/ml of streptomycin (Invitrogen, Barcelona, Spain). 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) 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 $50x10^6$ PBMC/ml in heat-inactivated FCS containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Madrid, Spain). 1 ml aliquots were frozen and conserved, until need, in liquid nitrogen. Monocytes and primary CD4+ T cells were purified from peripheral blood mononuclear cells by negative selection (StemCell, Vancouver, Canada). Macrophages were obtained as described before (Bosch et al., 2006). We cultured monocytes for 3 days with M-CSF (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) compound was performed as described before (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

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purchased from Sigma-Aldrich (Madrid, Spain), the oligonucleotide targeting gp120 Zintevir (AR177), the CXCR4 antagonists AMD3100 and ALX-40-4C and the fusion inhibitor peptide C34 were synthesized as described elsewhere (Armand-Ugon et al., 2003a; Doranz et al., 1997; Este et al., 1999a; Este et al., 1998; Murakami et al., 1997). The CCR5 antagonist TAK-779 reviewed in (Este, 2003), the RT inhibitors efavirenz, nevirapine and lamivudine (3TC), reviewed in (De Clercq, 2004) were received from the NIH AIDS reagent program and the monoclonal antibody anti-CCR5 PRO140 from Progenics Inc. 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.

Viruses

The HIV-1 strains BaL, HXB2, NL4-3 and 89.6 were obtained from the MRC Centre for AIDS Reagents (London, UK). The X4 strain J130.3 was kindly provided by Dr. O. T. Keppler. The HIV-1 NL4-3 strain that is resistant to T20/C34 has been described elsewhere (Armand-Ugon et al., 2003a; Menendez-Arias and Este, 2004). The IRLL98 HIV-1 strain contains the following mutations in the RT coding sequence: M41L, D67N, Y181C, M184V, R211K, T215Y (conferring resistance to NRTI) and mutations K101Q, Y181C, G190A (conferring resistance to NNRTI). HIV-1 strains K103N, Y181C, and Y188L, which have mutations conferring resistance to NNRTI, and HIV-2 ROD were obtained from the MRC Centre for AIDS Reagents (London, UK). 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 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 multiplicity of infection (moi) of 0.003 and exposed

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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 (Armand-Ugon et al., 2005; Armand-Ugon et al., 2003a). Anti-HIV activities were performed three times in triplicates. 50% effective concentrations (EC_{50}) were calculated as valid when the variation between replicates was less than 4-fold. Mean EC_{50} ± standard deviation (SD) for control compounds AZT and AMD3100 was 0.0008 ± 0.0002 µg/ml 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 EC_{50} value as compared to the wild type HIV-1 strain.

Anti-HIV activity in PMBC was determined as described (Moncunill et al., 2005): cells were incubated with each HIV-1 viral stock (200 $TCID_{50}/10^6$ cells) or mock-infected for 3 h at 37ºC, and thereafter were washed twice with phosphate buffered saline (PBS) 1x. Infected cells were seeded in 96-well plates ($0,15x10^6$ cells/well) and incubated 7-days at 37ºC, 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 PBS 1x. The cell death was quantified by flow cytometry in forward versus side scatter plots, as previously described. 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 SD were calculated for the control drugs AZT and AMD3100 against the NL4-3 wt strain. The EC_{50} 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 stimulation with M-CSF 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 the R5 strain BaL at a final concentration of 3700 pg/ml of HIV p24 antigen. At day 3, 7, 10 and 14 post-infection, 20 µl of culture supernatant were replaced by 20 µl of fresh complete medium with or

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without the corresponding drug. HIV production was analyzed at day 7 and 14 after infection by HIV p24 antigen detection in the culture supernatants (Innotest^{TM} HIV-Ag; Innogenetics, Barcelona, Spain).

Anti-HIV activity in lymphoid tissue was evaluated as described before (Glushakova et al., 1998; Grivel and Margolis, 1999). Tonsils from HIV-negative individuals from therapeutic tonsillectomy were maintained in PBS 1x, dissected into 2–3-mm blocks and placed on top of gelatine sponge gels (Espongostan, Prisfar) with RPMI 1640 10% FCS P/S. HIV-1 infection was carried out with 1.5 µl of the R5 HIV-1 BaL, the X4 NL4-3 and the dual tropic 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^{TM} HIV-Ag; Innogenetics, Barcelona, Spain).

Evaluation of cell death and HIV transfer

$2x10^5$ primary CD4 T cells 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 PBS 1x, fixed and permeabilized (Fix & Perm; Caltag, Burlingame, CA), and stained with KC57 anti-HIV capsid p24 antigen (CA p24) MAb (Coulter, Barcelona, Spain) and analyzed in a LSR II flow cytometer (BD, Madrid, Spain). Cells were identified by morphological parameters. Also 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 incubated for 1h at 20ºC in the presence or absence of test compounds (AR177, AMD3100, ALX-40-4C, POL3026, C34, T20 or AZT). Next, they were washed twice with cold PBS and compounds were added at various times post-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

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its EC_{50}). Virus production as quantity of p24 antigen in supernatant was determined 30 hours postinfection (Armand-Ugon 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 previously reported (Pauls et al., 2007). Briefly, 0.2×10^6 cells were washed in PBS and incubated for 20 minutes at room temperature with monoclonal antibodies (mAbs) anti-CD45 conjugated with fluorescein isothiocyanate (FITC), 12G5 (anti-CXCR4) phycoerythrin (PE), 2D7 (anti-CCR5) allophycocyanin (APC) and Leu3a (anti-CD4) peridin chlorophyll protein (PerCP) (BD, San Jose, CA) and with or without various drugs. The cells were then washed with PBS 1x and were fixed in PBS containing 1% formaldehyde and analyzed by flow cytometry in a LSR II system (BD, San Jose, CA). Data were acquired and analyzed with FacsDiva software (BD). 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 if differences in 12G5 mAb binding were due to CXCR4 down regulation, parallel experiments were done at 4ºC (30 min of incubation) and 37ºC (15 min of incubation). The IC_{50} of POL3026, AMD3100 and the chemokine SDF-1α was calculated for each condition.

Measurement of intracellular calcium concentrations

The intracellular calcium concentrations [Ca^{2+}] were determined as described previously (Este et al., 1999b). Briefly, CEM-CCR5 cells or THP-1 cells were loaded with Fluo-4 at a 2,5 μM (Sigma-Aldrich, Madrid, Spain). Fluorescence was measured in a Fluoroskan Ascent fluorometer (Labsystems, Helsinki, Finland). 1×10^6 cells were first stimulated with dilution buffer (control) or test compound at various concentrations. As a second stimulus, SDF-1α (100 ng/ml), RANTES, MIP-1α or MIP-1β (1000 ng/ml) were used to induce [Ca^{2+}] signaling. The second stimulus was added 120 seconds after the first stimulus.

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Chemotaxis assay.

The bottom chambers of HTS Transwell-96 chambers of 5-µm pore (Corning, UK) were filled with 150 µl of RPMI medium containing 20 ng/ml of the chemoattractant SDF-1α and different concentrations of POL3026 or AMD3100 and preincubated for 30 minutes at 37°C. Then, CEM-CCR5 cells (0,25 × 10^6 in 50 µl of RPMI 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 counted on a FACScalibur flow cytometer. Data is 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).

1,5x10^5 Sup-T1 cells were infected with 13 ng of p24 antigen from the HIV-1 R5 (non-syncytium inducing) 168.1 virus. To obtain a 168.1 stock, 5x10^6 Sup-T1cells were transfected with 2 µg proviral DNA using 0,4cm cuvettes (BioRad), and 250 V and 950 µF.

Parallel cultures with different inhibitory conditions were maintained. The starting concentration of each compound was determined by its EC_{50} in PBMC when tested against 168.1 virus. Twice a week the cultures were passed by splitting 1/5 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 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, Barcelona, Spain). 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 -80ºC for future phenotype analysis. Cell pellets were used for the genetic analysis of proviral forms.

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Development of resistant HIV-1

$0.1x10^6$ MT-4 cells were incubated with the HILV-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 new fresh MT-4 cells. The POL3026 concentration was progressively increased, finishing the passages when the concentration reached 0.034 μg/ml 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). The Expand High Fidelity PCR System from Roche and the 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, Barcelona, Spain). The *env* gene (5514-8910) was amplified with primers 5'-gataaagccacctttgcctagt-3' and 5'-ttctaggtctcgagatactg-3' as previously described (Armand-Ugon et al., 2005). Amplified DNA from the gp120 gene was sequenced with several primers (Armand-Ugon et al., 2005) with the ABI Prism™ BIGDYE Terminator 3.1 kit (Applied Biosystems, Madrid, Spain) and data was collected with the ABI Prism 3100 *Avant* Genetic Analyzer. Sequences were analyzed with the Sequencher 4.5 software and edited with the 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 moi 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, Barcelona, Spain). Triplicate values from days 1, 2, 3, 4 and 5 were obtained.

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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 while untreated infected cultures (the 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, 10% resistant plus 90% wt). Every 5-7 days, the supernatant was used to infect fresh MT-4 cells and aliquots of cells were harvested and stored at -80ºC for subsequent analysis. Detection of each virus population was assessed by sequencing the V3 loop of gp120 as explained above.

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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 throughput evaluation of candidate antiviral agents (Este, 2003; Menendez-Arias and Este, 2004). As shown in Table 1, POL3026 proved to be highly potent against several X4 HIV strains. An EC_{50} 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, efavirenz or the fusion inhibitor T-20 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). 50% cytotoxic concentrations (CC_{50}) of all compounds tested are shown in Table 1.

POL3026 is active against R5X4 and X4 strains in PBMC.

Dual-tropic (R5X4) HIV-1 strains preferentially use CXCR4 as entry coreceptor (Yi et al., 2005). POL3026 blocked the replication of R5X4 strains (3 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 as compared to the NL4-3 strain in peripheral blood mononuclear cells (PBMC). Also, POL3026 appeared to be less effective against the NL4-3 strain in PBMC than in MT-4 cells. Whilst this difference may be consequence of variation in the models used (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.

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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 cells expressing X4 HIV-1 glycoproteins but it did not prevent cell death induced by MOLT-4/BaL (R5) cells. 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 previously shown (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 that 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 post-infection. Virus production is measured after one cycle of replication. As shown in Fig 2B, similar to the CXCR4 agents AMD3100 or Alellix-4C, POL3026 began to loose its activity if addition was delayed for 15 min and kinetics of virus growth was different to 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 inhibits 12G5 mAb staining and SDF-1α-induced intracellular Ca2+ signaling and chemotaxis

In order 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 (IC_{50} of 0.0005 μg/ml) (Fig 3B). Conversely, POL3026 did not interfere with the staining by mAb specific for CCR5, CD45 or CD4 (data not shown). To determine if the inhibition of the CXCR4 mAb staining was due to a downregulation of CXCR4 or only a masking of the

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epitope recognized by the 12G5 antibody, we calculated the 50% inhibitory concentration (IC_{50}) of POL3026, AMD3100 and SDF-1α at 37ºC (both downregulation and epitope masking may occur) and at 4ºC (only masking of the epitope is evaluated). The IC_{50} for POL3026 (0.0067±0.005 µg/ml) and AMD3100 (0.0061±0.0022 µg/ml) at 37ºC did not change significantly at 4ºC (6-fold and 2-fold difference respectively). Conversely, the IC_{50} of the agonist chemokine SDF-1α (1.03 µg/ml) was 31-fold higher at 4ºC reflecting its capacity to downregulate and mask the 12G5 mAb epitope. To further evaluate the interaction of POL3026 with CXCR4 coreceptor, we tested its effect on chemokine induced intracellular Ca^{2+} signaling ($[Ca^{2+}]_i$). POL3026 by itself did not induce Ca^{2+} mobilization in CEM or THP-1 cells. POL3026 blocked Ca^{2+} 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 Ca^{2+} 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 downregulation and 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 than AZT and AMD3100. Conversely, no effect on R5

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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 was made resistant to AMD3100 albeit 200-fold loss in sensitivity. Similarly, the SDF-1α-resistant HIV-1 strain was also cross-resistant to POL3026 and AMD3100 (6-fold 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-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 a 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 as 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, most probably due to the lower replicating rate compared to 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

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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-fold and 25-fold resistant to POL3026 and 10-fold and 23-fold cross-resistant to AMD3100, respectively but 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 believed 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 impact on 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 to that of the HXB2.41 and the AMD3100-resistant virus shown to have reduced fitness (Armand-Ugon et al., 2003b) (Fig 5D). The growth of the HP41resA strain appeared 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.

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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 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-1α suggesting that POL3026 acts as an antagonist of CXCR4. Furthermore, multiparametric evaluation of HIV envelope function and time of addition experiments suggest that POL3026 blocks HIV replication at a time/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 with the same genetic background (i.e. NL4-3) but made resistant to other ligands of CXCR4. Cross-resistance may not be obligatory as ligands such as AMD3100, SDF-1a and POL3026 may interact differently with CXCR4 and 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} 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 clinical isolates showed a loss of sensitivity to POL3026 and AMD3100 compared to the NL4-3 strain in PBMC, probably due to their capacity to use both

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coreceptors. POL3026 did not inhibit the replication of R5 tropic virus but, it blocked the replication of macrophage tropic HIV-1 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 results it was not surprising the emergence and location of mutations developed under selective pressure with this compound. These mutations appear mainly in the V3 loop of gp120 and four mutations (Q310H, I320T, N325D, and A329T) are shared by viruses resistant to SDF-1α (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 indicate that HP38resB that is 25-fold resistant to POL3026 may have an impaired replication capacity. These results contrast to the marked reduced fitness of the AMD3100-resistant virus (Armand-Ugon 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 (Este 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 may coexist as a minor subpopulation that may be unnoticed by available methods of detection (Weber et al., 2006). In cell culture experiments, 3 of 6 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

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treated with a CCR5 agent, more patients had a change in tropism to dual tropic/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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Footnotes

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Figure 1. Structure of POL3026.

Figure 2. A. Effect of compounds (the RT inhibitor AZT, the fusion inhibitor C34, the anti-CD4 mAb Leu3A and the 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 SD. **B.** Effect of the addition of compounds (the binding inhibitor AR177, the CXCR4 antagonists AMD3100 and ALX-40-4C, POL3026, the fusion inhibitors C34 and T20 and the RT inhibitor AZT) at different moments after initiation of infection on the p24 production by NL4-3 in MT-4 cells 30h post-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.

Figure 3. A. Inhibition of mAb 12G5 (anti-CXCR4) staining on CEM-CCR5 cells by different compounds (the CXCR4 antagonist AMD3100 at 0.2 µg/ml, the anti-CCR5 mAb PRO140 at 10 µg/ml and POL3026 at 0.04 µg/ml). **B.** Dose-response curve of the inhibition of staining of mAb anti-CXCR4 12G5 by POL3026 (♦) and the CXCR4 antagonist AMD3100 (■); mAb anti-CCR5 2D7 by the anti-CCR5 mAb PRO140 (Δ) and mAb anti-CD4 Leu3a by unstained Leu3a (○). **C.** Calcium mobilization induced by SDF-1α (100 ng/ml) in $0.2x10^6$ CEM-CCR5 cells is blocked by POL3026 (1 µg/ml). Representative experiments are presented in panels A, B and C. The results were confirmed in three separated experiments. **D.** CEM-CCR5 cells induced to migrate through a 5-µm pore membrane by the CXCR4 ligand SDF-1α (20 ng/ml) in the presence or absence of POL3026 or the CXCR4 antagonist AMD3100 as control. Cell migration was quantified by flow-cytometry and expressed as migration index. Data shown is representative of experiments performed at least twice

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Figure 4. A. Inhibition of HIV-1 replication of J130.3 X4 strain in MDM. Concentrations of compounds used were TAK-779 (CCR5 antagonist), 1µg/ml; AMD3100 (CXCR4 antagonist), 5µg/ml; POL3026, 0,008µg/ml; AZT (RT inhibitor), 0,2µg/ml and C34 (fusion inhibitor), 2µg/ml. Graphic data are 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 tonsillary lymphocyte cultures. When tested, the represented concentrations of compounds were TAK-779, 1 µg/ml; AMD3100, 2 µg/ml; POL3026, 1 µg/ml and AZT, 1 µg/ml. A representative experiment performed in triplicates is shown. Similar results were obtained in three separated experiments (tonsil tissues coming from different donors). For A and B, values of p24 production at each point are represented relative to the p24 produced by the control culture without compound. Errors bars indicate SD. NC, no compound.

Figure 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 out 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, HP38resB 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 which emerged under different CXCR4 targeting compounds: POL3026, AMD3100 (de Vreese et al., 1996), the chemokine SDF-1α (Schols et al., 1998) and the T134 CXCR4 inhibitor (Kanbara et al., 2001).

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Table 1. Anti-HIV activity of POL3026

HIV Strain	Sub-type	Cells	EC$_{50}$ (µg/ml)[a] [Fold resistance [b]]								
			Compound								
			POL3026	AMD3100	SDF-1α	TAK-779	C34	T20	AZT	Nevirapine	Efavirenz
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	-

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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

[a] EC_{50}: Effective concentration 50 or the concentration needed to inhibit by a 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

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the mean of three separate experiments performed in triplicates. Standard deviation (SD) for control compounds AZT and AMD3100 was 0.0008 ± 0.0002 µg/ml and 0.001± 0.0004 µg/ml, respectively. The SD for all values is shown in an online supplementary table.

[b] CC_{50}: Cytotoxic concentration 50 or the concentration needed to induce 50% cell death in mock-infected MT-4 cells by the MTT assay.

[c] Fold Resistance: Relative loss of activity of the corresponding virus relative to the HXB2 wt strain calculated as the Mean EC_{50} of f the virus strain / Mean EC_{50} of the HXB2 wt strain.

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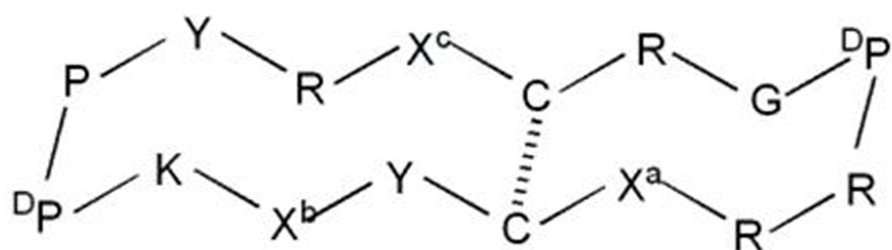
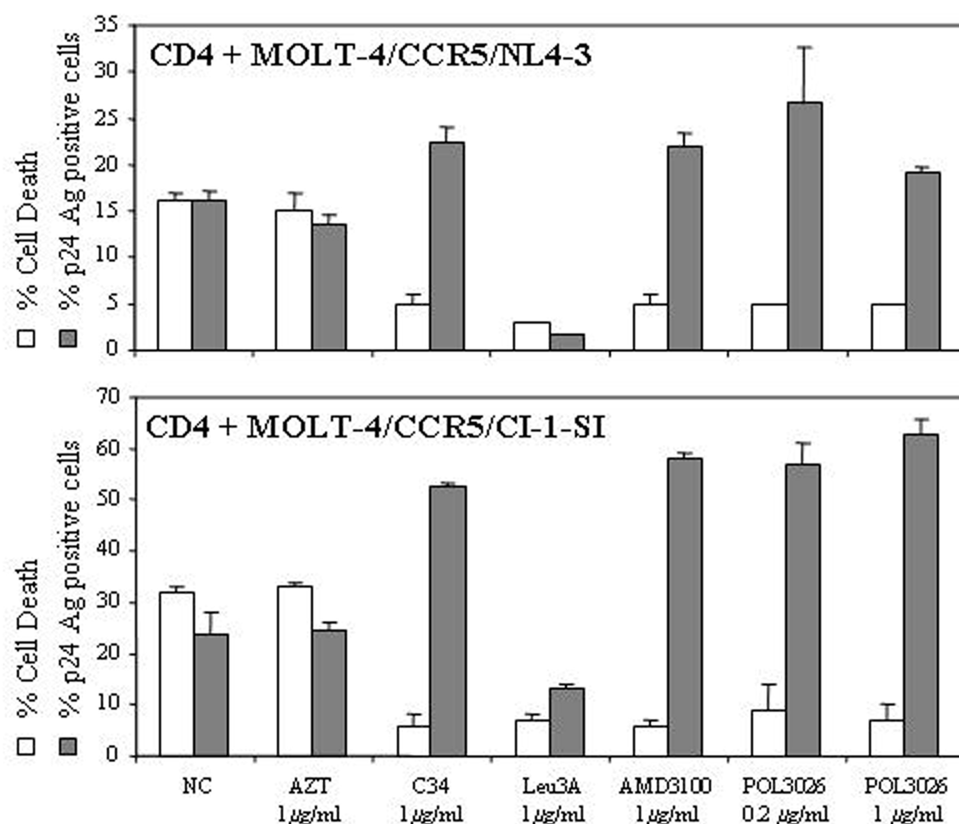
Figure 1



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

Figure 2

A



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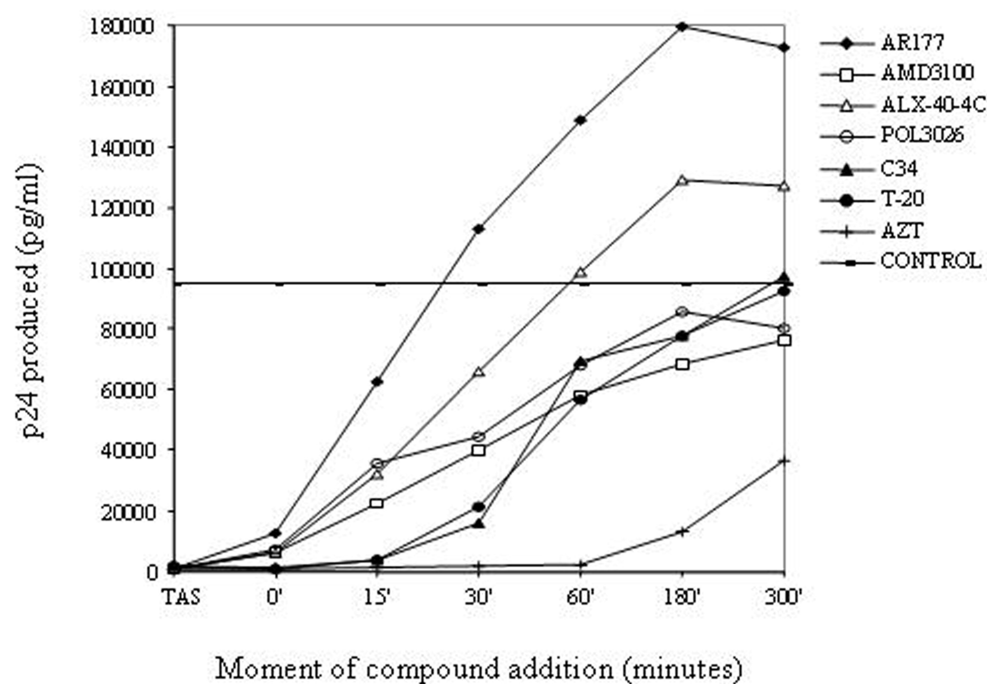
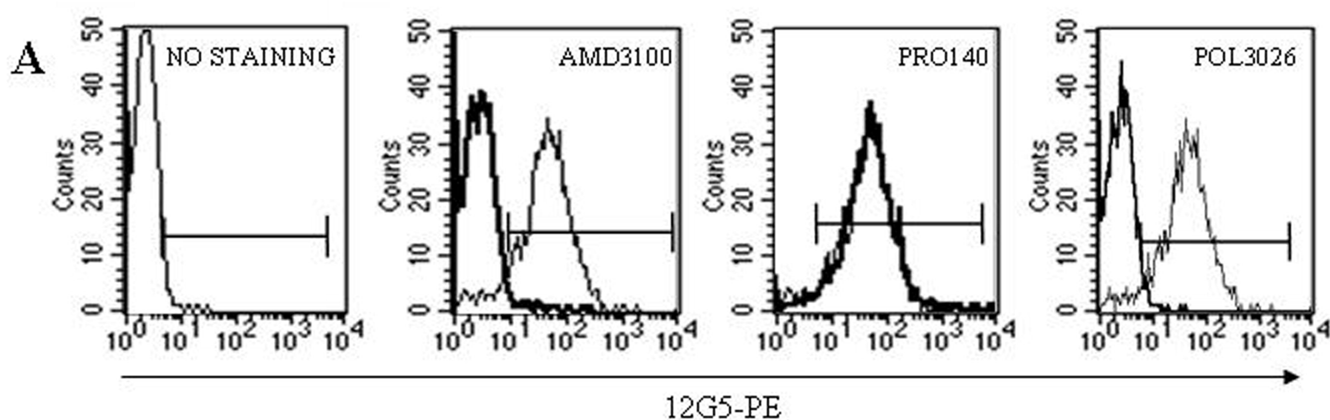


Figure 3

A



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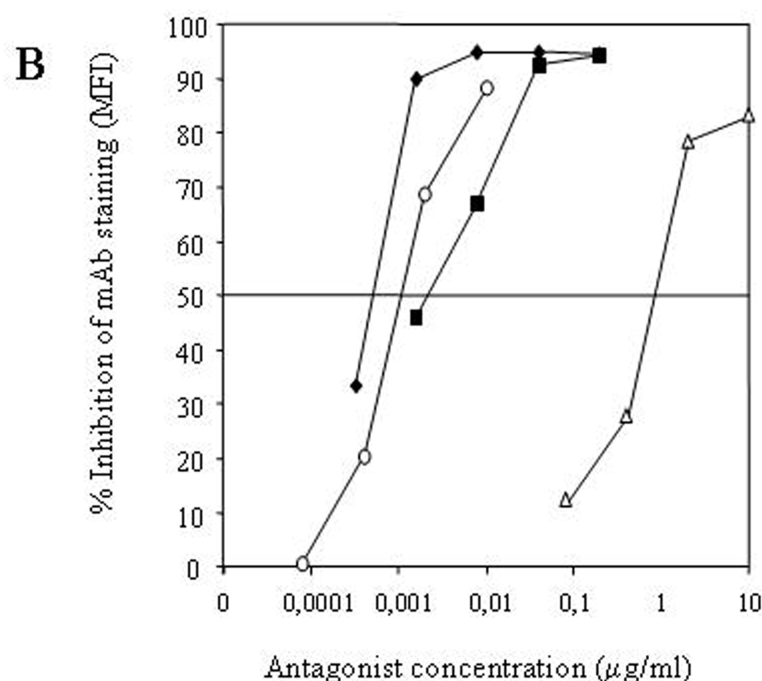

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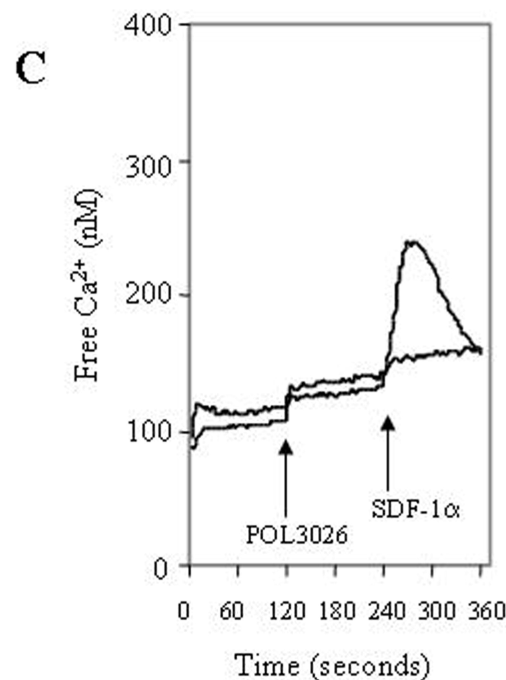

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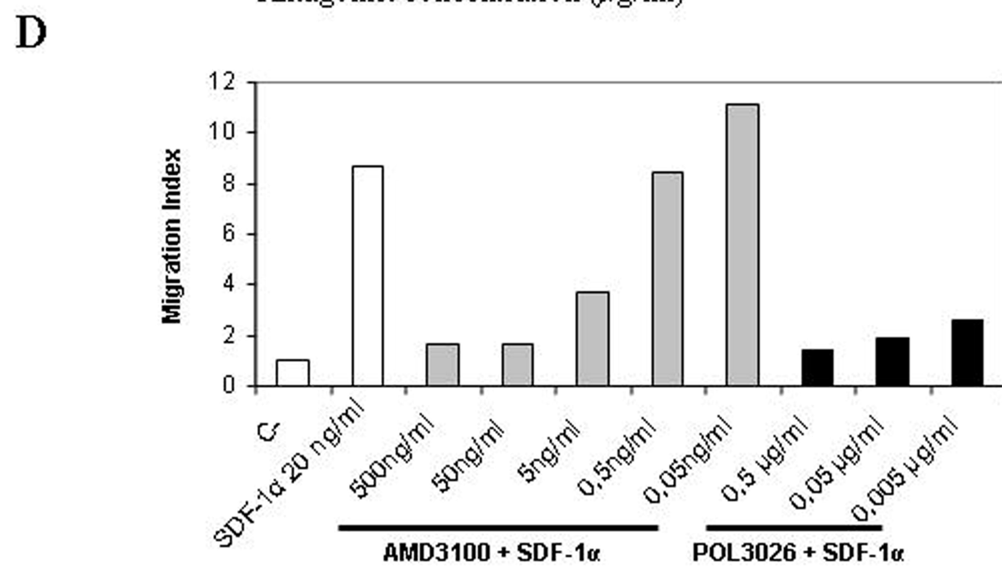


Figure 4

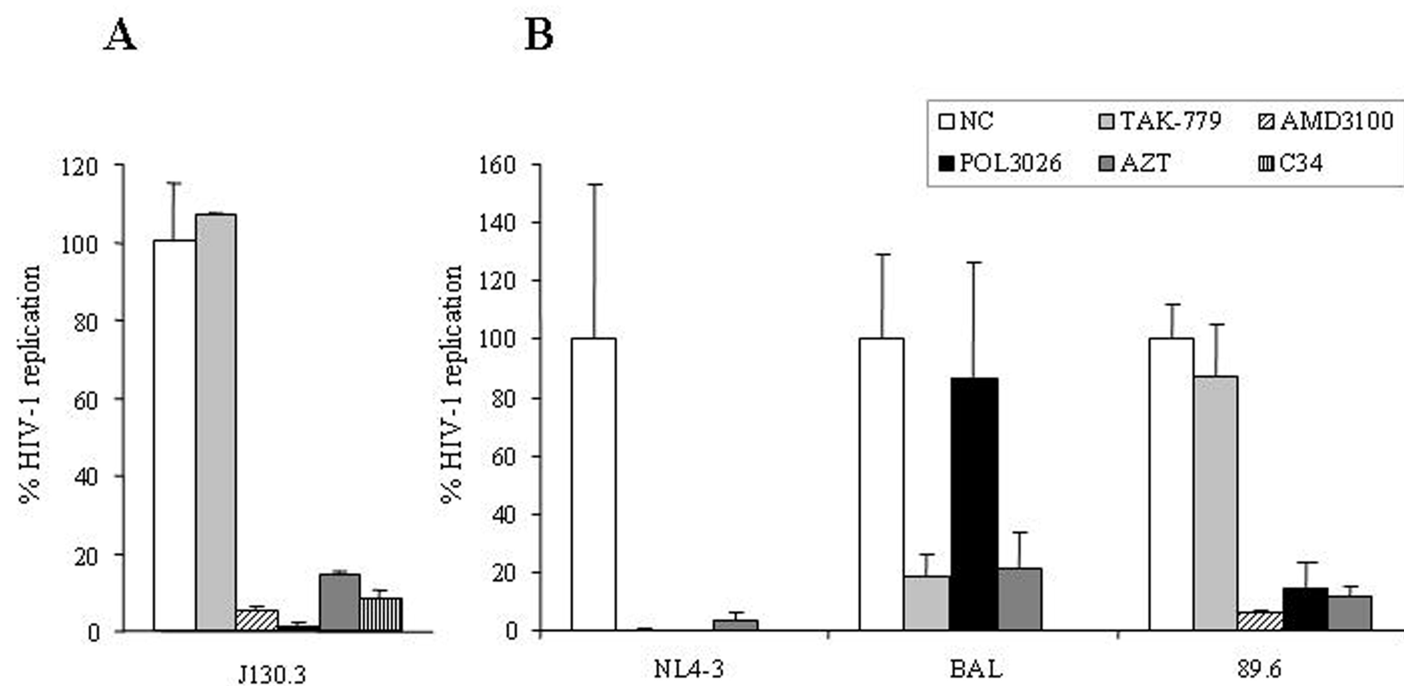
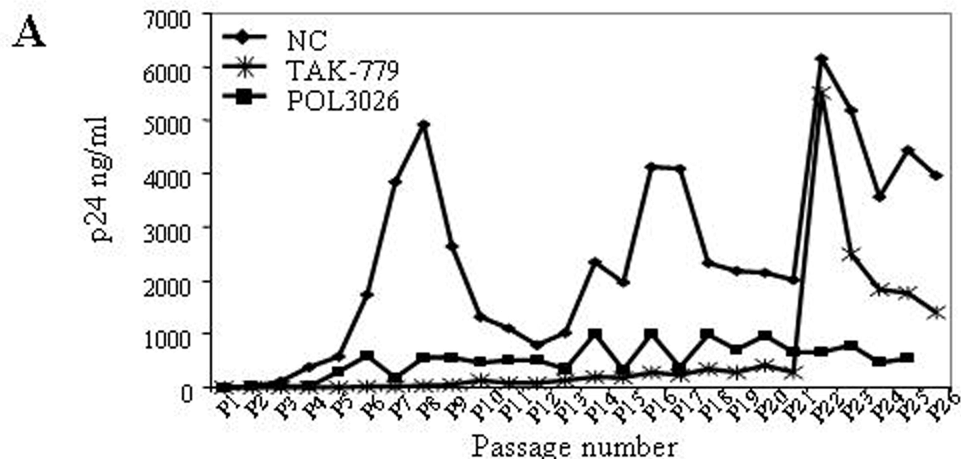


Figure 5

A



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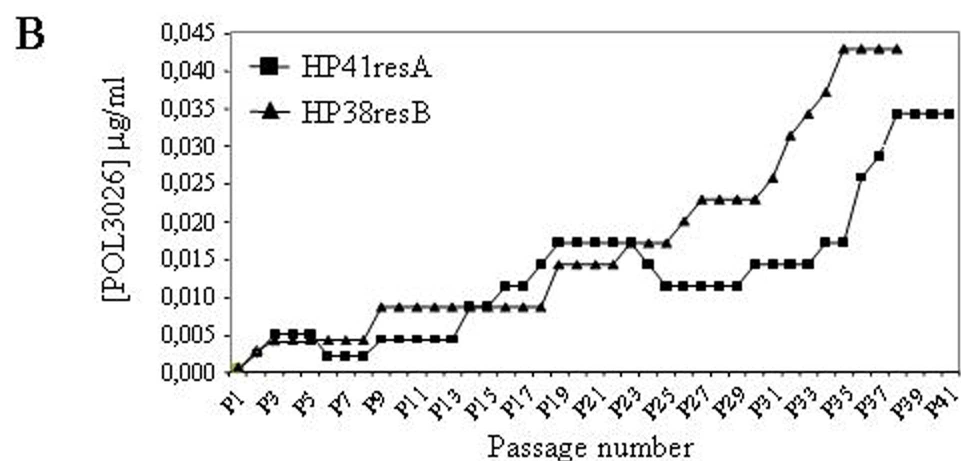

C

Virus	Mutations														
	C1	C2		V3					V4						C5
	96	200	232	310	316	320	325	329	396	397	398	399	400	417	473
HXB2wt	W	V	T	Q	A	I	N	A	F	N	S	T	W	P	G
HXB2.41	W	**I**	T	Q	A	I	N	A	F	N	S	T	W	P	**S**
HP41resA	**G**	V	**T/M**	Q	**T**	I	**D**	**T**	-	-	-	-	-	**L**	G
HP38resB	**G**	V	T	**H**	A	**T**	**D**	A	F	N	S	T	W	P	G

Figure 5 (ctnd)

D

2500
2000
1500
1000
500
0
p24 production (ng/ml)
HXB2wt
HP41resA
HP38resB
3100r
day 1 day 2 day 3 day4 day5

E

Virus strain	V3 Amino Acids																																				
HXB2	C	T	R	P	N		N	N	T	R	K	R	I	R	I	Q	R	G	P	G	R	A	F	V	T	I	G		K	I	G	N	M	R	Q	A	H	C
HP41resA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	D	-	-	-	T	-	-
HP38resB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	D	-	-	-	-	-	-
NL4-3	C	T	R	P	N		N	N	T	R	K	*S*	I	R	I	Q	R	G	P	G	R	A	F	V	T	I	G		K	I	G	N	M	R	Q	A	H	C
AMD3100r	-	-	-	-	-	N,Y	-	S	-	T		R	-	-	-	H	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	H	-	-	-	T	-	-
SDFr	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	D	-	-	-	-	-	-
T134r	-	-	-	-	-	-	K	-	-	-	-			-	-	T	K	-	-	-	-	V	L	Y	-	T	-	T	E	-	-	D	I	-	K	-	-	-