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Potential New Therapeutic Agents: Effects on HIV Replication and Viral Escape

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Dra. Paz Martínez Ramírez

Als meus pares

Al Javi

PUBLICATIONS

Pauls, E., Ballana, E., **Moncunill, G.**, Bofill, M., Clotet, B., Ramo-Tello, C. and Esté, J.A.**Evaluation of the anti-HIV activity of natalizumab, an antibody against integrin alpha4.** AIDS 2008 in press

Moncunill, G., Armand-Ugon, M., Clotet-Codina, I., Pauls, E., Ballana, E., Llano, A., Romagnoli, B., Vrijbloed, J., Gombert, FO., Clotet, B., De Marco, S., Esté, JA. **Anti-HIV Activity and Resistance Profile of the CXCR4 Antagonist POL3026.** Mol Pharmacol. 2008 Apr;73(4):1264-73.

Moncunill, G., Armand-Ugon, M., Pauls, E., Clotet, B. and Esté, J.A.
HIV-1 Escape to CCR5 Coreceptor Antagonism through selection of CXCR4-using variants *in vitro*. AIDS. 2008 Jan 2;22(1):23-31.

Senserrich, J., Pauls, E., Clotet-Codina, I., Armand-Ugón, M., **Moncunill, G.**, Clotet, B. and Esté, J.A. **HIV-1 Resistance to the Anti-HIV Activity of a shRNA Targeting a Dual-Coding Region.** Virology. 2008 Mar 15; 372(2):421-9.

Moncunill, G., Negredo, E., Bosch, L., Vilarrasa, J., Witvrouw, M., Llano, A., Clotet, B., Esté, JA. **Evaluation of the Anti-HIV Activity of Statins.** AIDS. 2005 Oct 14;19(15):1697-700.

ABREVIATIONS

AIDS	acquired immunodeficiency syndrome
APC	allophycocyanin
APL	aplaviroc
AZT	3'-azido-3' deoxythymidine
BSA	bovine serum albumin
CA	capside (p24)
CC_{50}	cytotoxic concentration 50%
CD	cluster of differentiation
CI	clinical isolate
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
DNA	deoxuribonucleic acid
DS	dextran sulfate
EC_{50}	effective concentration 50%
ECL	extracellular loop
ELISA	enzyme-linked immuno sorbent assay
Env	envelope
FCS	fetal calf serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FSC	forward scatter
Gag	group specific antigen
Gp120	glycoprotein 120
Gp41	glycoprotein 41
HAART	highly active antiretroviral therapy
HDL	high density lipoprotein
HIV	human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HR	heptad repeat
IC_{50}	inhibitory concentration 50%
Ig	immunoglobuline

IL-2	interleukine-2
IN	integrase
LDL	low density lipoprotein
LTNP	long-term non-progressor
LTR	long terminal repeat
MA	matrix protein (p17)
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MIP	macrophage inflammatory protein
MHC	major histocompatibility complex
MOI	multiplicity of infection
MRC	Medical Research Council
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVC	maraviroc
MW	molecular weight
NC	nucleocapsid (p7)
NIH	National Institutes of Health
NNRTI	non-nucloside analogue reverse transcriptase inhibitors
NRTI	nucleoside analogue reverse transcriptase inhibitors
NSI	non-syncytium inducing
Nt	amino-terminus, N-terminus
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin cholorohyll protein
PHA	phytohemagglutinin
PI	propidium iodide
Pol	polymerase
PR	protease
RANTES	regulated on activation, normal Tcell expressed and secreted
RNA	ribonucleic acid
RT	reverse transcriptase
SD	standard deviation

SDF-1	stromal cell-derived factor 1
SI	syncytium formation
SIV	simian immunodeficiency virus
SSC	side scatter
SU	surface (glycoprotein gp120)
TCID	tissue culture infectious dose
TM	transmembrane (glycoprotein gp41 or domain)
UNAIDS	United Nations Programme on HIV/AIDS
VL	viral load
Wt	wild-type

SUMMARY

Developing new anti-HIV compounds targeting different steps of the HIV cycle is a continuous need due to the emergence of drug-resistant strains and also to the long-term toxicity of current antiretrovirals used for treatment. HIV entry requires the binding of the viral particles to the CD4 receptor and a coreceptor through the viral envelope glycoprotein gp120, triggering structural changes in gp41 that promote the fusion of the viral and host cell membranes and viral core release into cells. HIV strains can be classified into different tropisms depending on which coreceptor they use: CCR5 (R5), CXCR4 (X4) or both coreceptors (R5X4). Therefore, HIV entry is an essential step that offers several potential new targets for antiviral agents. From the compounds developed, coreceptor antagonists are among the most promising agents. Another class of interesting compounds are statins, which are a well-established class of drugs prescribed for treatment of hypercholesterolemia. Recent studies suggest that statins have direct anti-HIV effects both *in vitro* and *in vivo* targeting HIV entry and budding. The impact on HIV of both classes of compounds and therefore, its consequences in long-term treatment are still unknown. We wanted to characterize statins as antiretroviral agents, determine the role of coreceptor inhibitors in the evolution of HIV tropism and characterize the novel CXCR4 antagonist POL3026. Unfortunately, we could not detect a significant anti-HIV activity of statins due to a high cytotoxicity in cell culture or any effect of simvastatin in a pilot study with 12 HIV+ patients after 8-12 weeks. Using an *in vitro* model to study coreceptor switch of R5 strains to R5X4 or X4, we found that the probability to change coreceptor use was dependent on the clinical isolate and also on the cell-culture conditions such as availability of CCR5. We observed that selective pressure of an anti-HIV compound can modify the evolution of coreceptor use. Reverse transcriptase (RT) inhibitors and CCR5 targeting agents delayed the emergence of CXCR4-using variants compared to untreated cultures. However, CXCR4-using variants emerged faster under CCR5 drug pressure than under RT inhibitors, whereas CXCR4 antagonists could prevent its emergence. We also characterized POL3026, which proved to be a potent anti-HIV agent against naïve and drug-resistant strains of X4 and R5X4 phenotype. Several assays and development of resistance allowed us to confirm that POL3026 blocked HIV replication through specific interaction with CXCR4. In conclusion, our results caution on the use of statins to treat HIV infection and on the risk that HIV may escape from CCR5 antagonists by selecting CXCR4 emerging variants. On the other hand, potent and specific CXCR4 antagonists are interesting antiviral agents that could prevent the emergence of R5X4 or X4 viruses.

RESUM

El desenvolupament de nous compostos anti-VIH és una continua necessitat degut a l'aparició de soques resistents i a la toxicitat a llarg terme dels fàrmacs actuals. L'entrada del VIH a les cèl·lules requereix la unió de les partícules virals al receptor CD4 i a un coreceptor a través de la glicoproteïna viral de l'embolcall gp120, induint canvis conformacionals a gp41 que promouen la fusió de la membrana viral i de la cèl·lula hoste. Les soques de VIH es poden classificar en diferents tropismes segons quin coreceptor utilitzen: CCR5 (R5), CXCR4 (X4) o ambdós coreceptors (R5X4). Així doncs, el procés d'entrada és un pas essencial que ofereix noves dianes per agents antivirals. Dels compostos desenvolupats, els antagonistes dels coreceptors són dels agents més prometedors. Una altra classe de compostos interessants són les estatines, una classe de fàrmacs ben establerts pel tractament de la hipercolesterolèmia. Estudis recents suggereixen que les estatines tenen efectes directes contra el VIH tant *in vitro* com *in vivo,* inhibint l'entrada i la gemmació de virions. L'impacte d'ambdues classes de compostos sobre el VIH i les seves conseqüències en els tractaments a llarg terme són encara desconeguts. Vam voler caracteritzar les estatines com a agents antiretrovirals, determinar el paper que juguen els antagonistes dels coreceptors en l'evolució del tropisme del VIH i caracteritzar el nou antagonista de CXCR4 POL3026. Malauradament, no vam poder detectar una activitat antiviral significativa amb cap de les estatines avaluades, degut a la gran citotoxicitat en els cultius cel·lulars. Tampoc vam poder observar cap efecte de la simvastatina en un estudi pilot en 12 pacients VIH+ durant 8-12 setmanes. Utilitzant un model *in vitro* per estudiar el canvi de coreceptor de soques R5 a R5X4 o X4, vam observar que la probabilitat de canviar depèn de l'aïllat clínic i també de les condicions de cultiu, com la disponibilitat de CCR5. La pressió selectiva d'un compost anti-VIH podia modificar l'ús coreceptor. Agents contra CCR5 i els inhibidors de la transcriptasa inversa (RT) endarrerien l'emergència de variants R5X4 o X4. Tot i això aquestes variants apareixien més ràpid sota pressió dels agents dirigits contra CCR5 que dels inhibidors de la RT, mentre que antagonistes de CXCR4 podien prevenir la seva aparició. Hem caracteritzat POL3026, que ha demostrat ser un potent inhibidor de soques salvatges y resistents a fàrmacs actuals amb fenotip X4 o R5X4. Diferents assajos i el desenvolupament de virus resistents ens ha permès confirmar que POL3026 bloqueja la replicació del VIH mitjançant la interacció específica amb CXCR4. En conclusió, els nostres resultats demanen cautela en l'ús de les estatines per tractar la infecció del VIH i en el risc de que el VIH pot escapar dels antagonistes de CCR5 mitjançant la selecció de virus que utilitzin CXCR4. Per una altra banda, els antagonistes de CXCR4 són interessants agents antivirals que podrien prevenir l'aparició de virus X4 o R5X4.

RESUMEN

El desarrollo de nuevos compuestos anti-VIH es una continua necesidad debido a la aparición de cepas resistentes y a la toxicidad a largo plazo de los fármacos actuales. La entrada del VIH a las células requiere la unión de las partículas de VIH al receptor CD4 y a un coreceptor a través de la glicoproteina viral de la envuelta gp120, induciendo cambios conformacionales en gp41 que promueven la fusión de la membrana viral y de la célula huésped. Las cepas de VIH se pueden clasificar en distintos tropismos dependiendo de que correceptor utilicen: CCR5 (R5), CXCR4 (X4) o ambos (R5X4). Por esto, el proceso de entrada es un paso esencial que ofrece nuevas dianas para agentes antivirales. De los compuestos desarrollados, los antagonistas de los correceptores son de los más prometedores. Otra clase interesante son las estatinas, fármacos bien establecidos para el tratamiento de la hipercolesterolemia. Estudios recientes sugieren que las estatinas tienen efectos anti-VIH tanto *in vitro* como *in vivo,* inhibiendo la entrada y la gemación de viriones. El impacto de ambas clases de compuestos sobre el VIH y sus consecuencias a largo plazo son aún desconocidos. Quisimos caracterizar las estatinas como agentes antivirales, determinar el papel de los inhibidores de los correceptores en la evolución del tropismo del VIH y caracterizar el nuevo antagonista de CXCR4 POL3026. Desgraciadamente, no pudimos detectar una actividad anti-VIH significativa con ninguna de las estatinas evaluadas, debido a una gran toxicidad en los cultivos celulares. Tampoco detectamos ningún efecto de la simvastatina en un estudio piloto con 12 pacientes VIH+ durante 8-12 semanas. Utilizando un modelo *in vitro* para estudiar el cambio de correceptor de cepas R5 a R5X4 o X4, observamos que la probabilidad de cambio depende del aislado clínico y de las condiciones de cultivo, como la disponibilidad de CCR5. La presión selectiva de un compuesto anti-VIH puede modificar la evolución del uso de correceptor. Agentes contra CCR5 e inhibidores de la transcriptasa inversa (RT) retrasavan la aparición de variantes R5X4 o X4. Aúnque estas variantes aparecían antes bajo presión de los agentes dirigidos a CCR5 que de los inhibidores de la RT, mientras que antagonistas de CXCR4 podían impedir su aparición. Hemos caracterizado POL3026 que ha demostrado ser un potente inhibidor de cepas salvajes y resistentes a fármacos actuales con fenotipo X4 o R5X4. Diferentes ensayos y el desarrollo de resistencias nos ha permitido confirmar que POL3026 inhibe la replicación del VIH mediante la interacción específica con CXCR4. En conclusión, nuestros resultados advierten del uso de las estatinas para tratar la infección del VIH y del riesgo de que el VIH pueda escapar de los antagonistas de CCR5 seleccionando cepas R5X4 o X4. Por otro lado, los antagonistas de CXCR4 son interesantes agentes antivirales que podrían prevenir la aparición de los virus que utilizan CXCR4.

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INTRODUCTION

HIV

The Human Immunodeficiency Virus (HIV) is the etiologic cause of the acquired immunodeficiency syndrome (AIDS) [1-3]. The infection with HIV results in the progressive qualitative and quantitative deterioration of the T lymphocyte subpopulation that expresses the CD4 receptor, leading to immune deficiency [4]. AIDS could be defined by the signs, symptoms, infections and cancers associated with the deficiency of the immune system from infection with HIV.

The estimated number of persons living with HIV worldwide in 2007 was 33.2 million, 2.5 million of people were newly infected and 2.1 million of people died from AIDS. Every day, over 6800 persons become infected with HIV and over 5700 persons die from AIDS, mostly because of inadequate access to HIV prevention and treatment services. The HIV pandemic remains the most serious of infectious disease challenges to public health (http://www.unaids.org/).

HIV is a member of the genus Lentivirus, part of the family of Retroviridae. There are two types of HIV: HIV-1 and HIV-2. HIV-1 is the cause of the majority of HIV infections in the World, whereas HIV-2 is confined to West Africa. HIV-1 is subdivided in different groups (M, N and O) [5]. Group M is the main cause of the pandemic of HIV-1 and it has several subtypes (A-H), being subtype B the predominant in Europe, America, Australia and in the major part of Asia. Whereas in Africa subtypes A and C are the more extended (reviewed in [5,6]).

Virion structure

The HIV mature virion is a spherical particle of 145 ± 25 nm of diameter with an envelope that contains an inner layer and a cone-shaped protein capsid (Fig. 1A and B) [7]. The HIV genome has two copies of single-stranded RNA of positive polarity and is enclosed by the capsid, which is formed by the viral p24 protein [7] typical from lentivirus. The RNA is tightly bound to the p7 protein from the nucleocapsid and enzymes such as the reverse transcriptase (RT), the protease (P) and the integrase (IN). Inside the capsid there are also the accessory proteins Vif, Vpr and Nef. Enclosing the capsid, there is a matrix formed by the p17 protein and it is surrounded by the envelope, a phospholipidic bilayer coming from the plasmatic membrane of host cells. It has

embedded viral glycoproteins in form of spikes. Those spikes are composed by the viral glycoproteins gp120 (surface glycoprotein) and gp41 (transmembrane glycoprotein) [8].

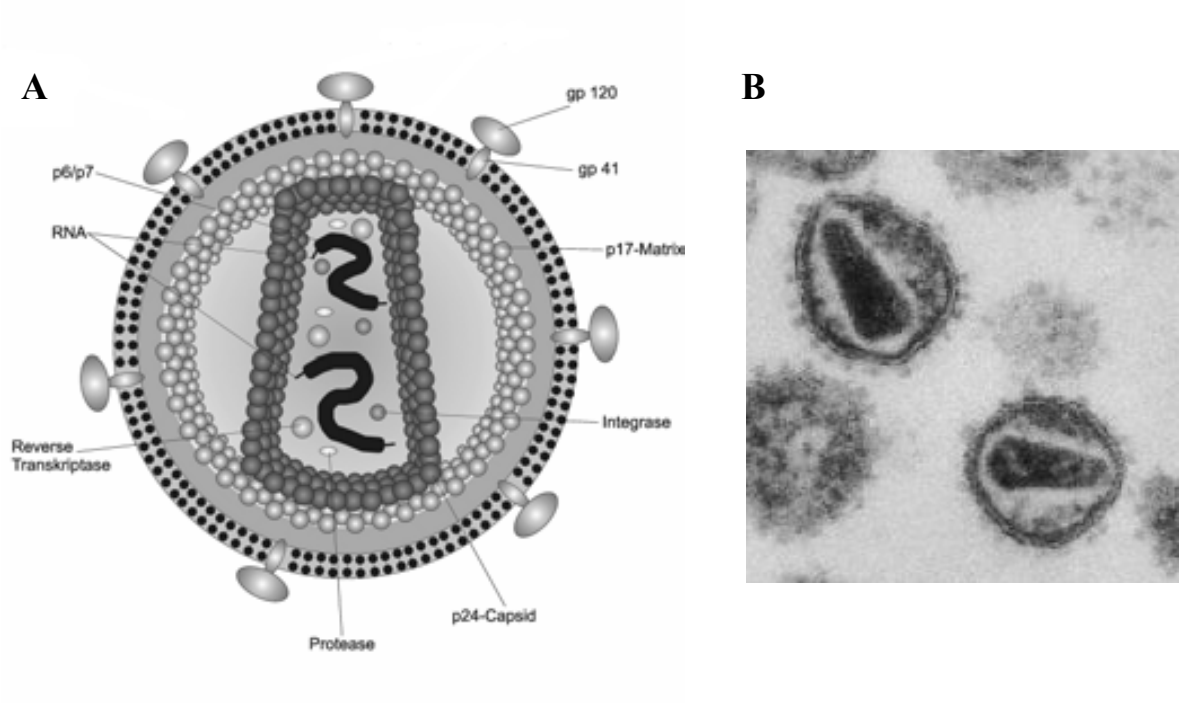


Figure 1. Structure of HIV-1 particles. (A) Schematic structure of an HIV particle (http://www.pipelinedrugs.com/biotechnology_encyclopedia/hiv.htm). **(B)** Electro micrograph of HIV-1 particles (d'Otago School of Medical Sciences)

Genome

The integrated form of HIV-1, also known as the provirus, is approximately 9.8 kilobases (kb) in length [9]. Both ends of the provirus are flanked by a repeated sequence known as the long terminal repeats (LTRs), which are required for the provirus to integrate to the cell-host DNA and they have binding sites for the transcription factors necessary to express the viral genes.

The RNA genome consists of nine genes (Fig. 2)[10]. Three of these genes (*gag, pol and env*) encodes polyproteins Gag, Pol and Env that are common between all retroviruses. Cleavage of those polyproteins give structural proteins: Gag proteins are p24 (capsid), p6 and p7 (nucleocapsid) and p17 (matrix protein); Pol cleavage results in

the viral enzymes necessary for replication, the RT (a dimer of P66/55), PR (p11) and IN (p32); and Env products are the viral envelope glycoproteins gp120 and gp41.

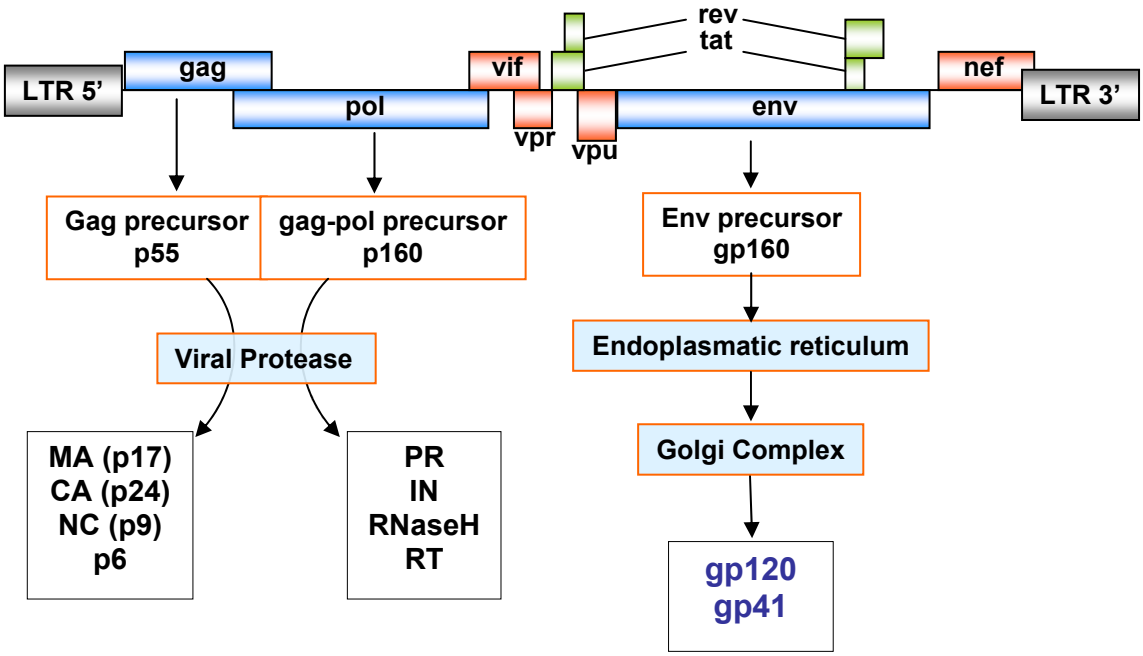


Figure 2. HIV genome organization (Armand-Ugón, M.)

The six remaining genes encode two regulatory proteins (*tat and rev genes*) and four accessory proteins (*vpu or vpx* for HIV-2*, vpr, vif and nef genes*) which affect the viral replication and infectivity in different ways [11]: *tat* encodes two proteins (p16 and p14) which are transcriptional transactivators for the LTR promoter binding the TAR RNA element [12]. Rev protein (p19) is involved in shuttling RNAs from the nucleus and the cytoplasm by binding to the RRE RNA element [11]. Vif protein (p23) is associated with viral infectivity, it prevents the action of APOBEC3G (a cell protein which deaminates DNA:RNA hybrids and/or interferes with the Pol protein) [13]. Vpu protein (p16) influences the release of new virus particles from infected cells [11]. Vpr (p14) arrests cell division at G2/M and facilitates the localization of the preintegration complex to the nucleus. And Nef protein (p27), which downregulates CD4, the major viral receptor and MHC class I molecules. Nef also interacts with SH3 domains [11].

Life cycle

HIV life cycle is divided in two phases: the early, regulatory phase, from virus entry to provirus integration in the genome, and the late, structural phase, from transcription of viral genes to delivery of new particles formed (Fig. 3).

The early stage begins when the virus binds to the cell surface, fuses with the cell membrane and the HIV capsid is released into the cytoplasm, where uncoating and release of viral nucleic acid take place. Next, the HIV reverse transcriptase enzyme copies the single-stranded (+) RNA into double-stranded DNA. This DNA is included in the pre-integration complex [14] which is then transported into the cell nucleus by Vpr. There, the integration of the viral DNA into the host cell genome is carried out by the viral enzyme integrase [15]. This integrated viral DNA may then lie latent for long periods of time [16,17].

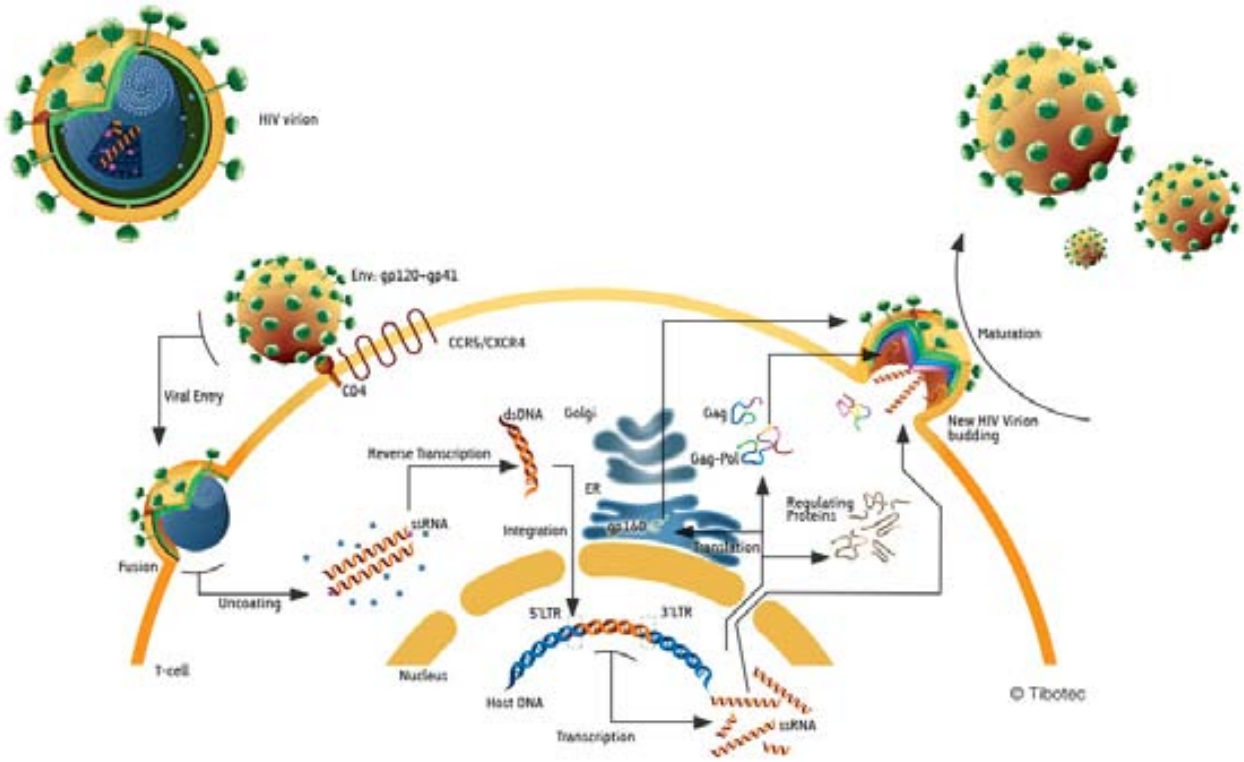


Figure 3. HIV replication cycle (adapted from www.tibotec.com)

In the late phase of the cycle, there is the transcription of the proviral DNA using cellular machinery, process trans-activated by Tat. The resulting RNA is spliced into different transcripts: whole RNA molecules (genomic-length) and RNA molecules that have been spliced one or several times [18]. Rev mediates the transport of those RNAs to the cytoplasm where the different viral protein precursors are translated and directed to the plasmatic membrane. Assembly of those protein precursors together with two molecules of viral RNA will end with the budding of new viral particles in the regions of the membrane that expresses Env [19]. Finally, the protease induces the proteolysis of gag-pol precursor obtaining mature viral particles with the capacity of beginning new infectious cycles [20].

The clinical course of infection

HIV-1 infection begins with transmission either by mucosal or parenteral exposure to the viruses. Natural progression of HIV-1 infection *in vivo* is associated with a progressive decrease of the $CD4^+$ T cell count and an increase in viral load (VL, copies of viral RNA/ml of plasma), but it can be divided in three phases.

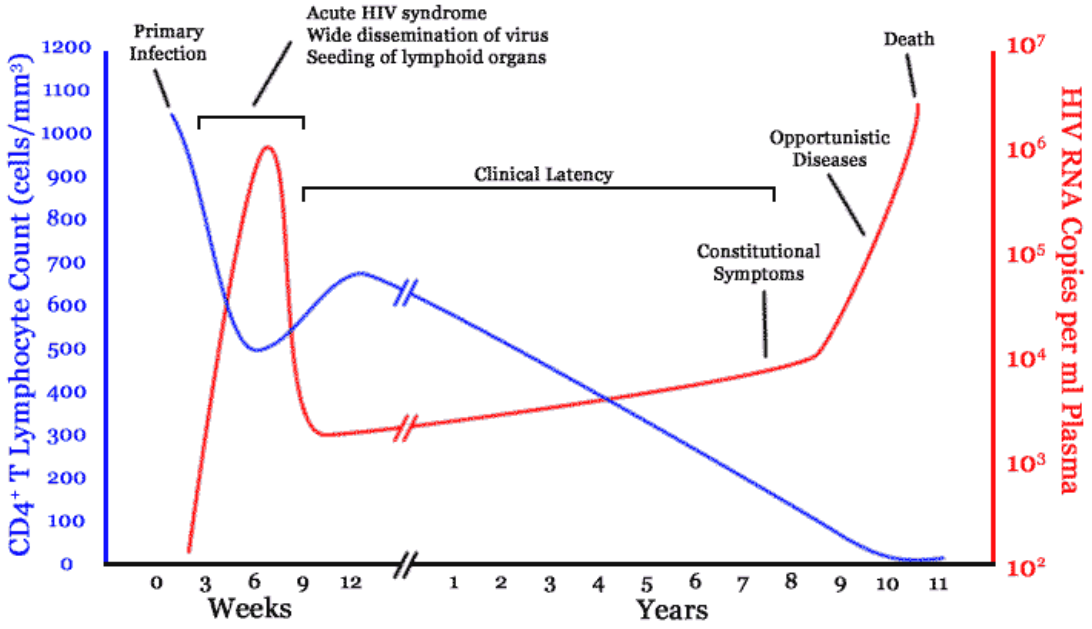


Figure 4. Graph showing HIV viral load and CD4+T cell levels over the course of an untreated infection [21]

Acute infection

The first stage of infection, the primary or acute infection, lasts between 2 and 4 weeks. It is a period of rapid viral replication that immediately follows the individual's exposure to HIV, leading to a peak in the amount of virus in plasma that reaches a VL of several million copies/ml. This allows the virus to spread through the body [22], but is accompanied by a strong immune response [23] that partially controls virus expansion and induces a decrease in VL. This acute viremia is associated with the activation of $CD8^+$ T cells, which kill HIV-infected cells, and subsequently with antibody production or seroconversion. CD4+ T cell count presents a moderate decrease during this phase but returns to normal levels (around 800 cells/µl, whereas the normal blood value is 1200 cells/µl). During this stage, the patient remains asymptomatic or with mild symptoms, similarly to other infection processes.

Chronic asymptomatic infection

This stage may last several years and is characterized to be an asymptomatic phase, in which the immune system can maintain the viral load to low levels. However, there is continuous viral replication. Virologic set point reached at this moment is a good marker of the posterior rate of disease progression [24]. There is also a sustained depletion of CD4+ T cells that induces the proliferation of new T cells. However this continuous proliferation and the activation of the immune system finally leads to a progressive immunodeficiency that takes to the last phase of the infection.

Advanced disease and AIDS

This phase is characterized by low numbers of CD4+ T cells and by an exhaustion of the immune system, so it is unable to replace the cells that are destroyed [25]. CD4 count falls to less than 200 cells/ml, while the virus amount in plasma increases [26]. There is a failure of immune system that allows for the appearance of opportunistic infections and cancers that threaten health and even life.

Before effective treatments were available, the average time for the progression to death was about 10 years for most patients [27]. However, a few of them called long-term non-progressors (LTNP) remain asymptomatic with no signs of disease progression in the absence of treatment for at least 10 years.

HIV ENTRY

The process of viral entry is a sequential process that requires specific interaction of the envelope with specific cell surface receptors and involves fusion of the viral envelope with the host cell membrane.

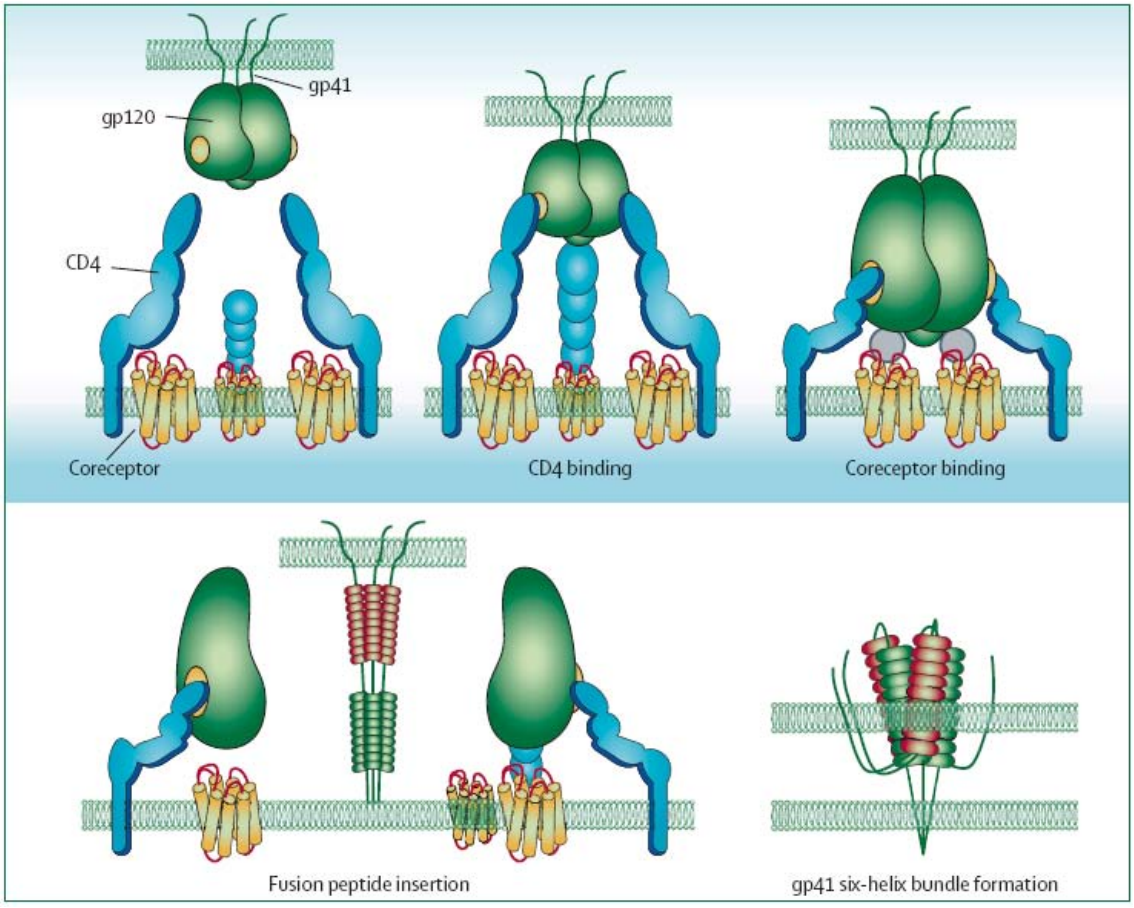


Figure 5. **Mechanism of HIV entry [28]**

The first event is attachment of the virus to host cell surface, which is rather unspecific and can be facilitated by host cell proteins present in both the viral envelope and target cell surface [29]. Next, trimeric gp120 on the surface of the virion binds to CD4 receptor on the surface of the target cell, inducing a conformational change that allows the binding of the gp120 through V3 to a coreceptor, mainly CCR5 or CXCR4. This interaction triggers structural changes in gp41. Assembled as a trimer, this coiled-coil protein, projects three peptide fusion domains that "harpoon" the lipid bilayer of the target cell. The fusion domains then form hairpin-like structures that draw the virion

and cell membranes together to promote fusion [30], leading to the release of the viral nucleocapsid into the cell cytoplasm.

Lipid rafts are membrane domains enriched in cholesterol and sphingolipids that are though to play a role in HIV-1 entry as well as in other stages of HIV-1 replication cycle [31]. For instance, lipid rafts have been suggested to aggregate CD4 and the HIV coreceptors, necessary for viral entry. Therefore HIV-1 entry requires adequate cholesterol levels in both host cell and viral membranes. Also, an actin cytoskeleton rearrangement seems to be necessary for the entry step [32].

HIV receptors

In 1984, the CD4 molecule was identified as necessary for HIV-1 replication within host cells [33]. Later studies showed that CD4 alone was not enough for HIV-1 infection of a host cell and then the chemokine receptor CXCR4 [34] and later, CCR5 [35] were identified as major coreceptors.

CD4

CD4 is a transmembrane glycoprotein of the immunoglobulin superfamily. It is mainly expressed as a 55 kDa monomer, but CD4 dimers and tetramers (110 and 220 kDa) have also been found to be expressed at the cell surface of T helper cells, regulatory T cells, monocytes, macrophages, and dendritic cells. The CD4 receptor normally functions as a co-ligand and coreceptor of the major histocompatibility complex class II (MHC II) molecule during T cell recognition of a foreign antigen, where it stabilizes the MHC II-peptide-T-cell receptor complex and initiates intracellular signal transduction leading to T cell activation [36]. The extracellular portion of CD4 is composed of four immunoglobulin (Ig)-like domains, designated D1 to D4, being D4 the membrane proximal domain. CD4 uses its D1 domain to interact with the β_2-domain of MHC class II molecules.

CD4 is the primary receptor used by HIV-1 to gain entry into host T cells [33]. The HIV-1 particles bind to CD4 with the envelope glycoprotein gp120 through the D1 domain [37], leading to the conformation change of the viral gp120 protein that allows the binding to the coreceptor. Nevertheless, CD4-independent infections have been

described for SIV [38], HIV-1 [39,40], HIV-2 strains [41] and also some clinical isolates.

Coreceptors: The chemokine receptors CCR5 and CXCR4

Chemokine receptors have a seven transmembrane structure and are coupled to G-protein. The ligands for these receptors are chemokines, which are small molecules (8 - 10 kD) in the cytokine family that promote chemotaxis and cellular activation. Following interaction with their specific chemokine, an intracellular signaling cascade is initiated via a G-protein release from the intracellular domain of the receptor [42] and triggers a flux in intracellular calcium ions (Ca^{2+}) (calcium signaling). The N-terminus of the receptor is extracellular and participates in binding of their ligands, whereas the C-terminus is intracellular and serves as the site for β-arrestin binding. β-arrestins facilitate a G-protein independent cell signaling through binding the chemokine receptor to clathrin for endocytosis and subsequent recycling [42-44].

Chemokine receptors are divided into different families, CXC chemokine receptors, CC chemokine receptors, CX3C chemokine receptors and XC chemokine receptors that correspond to the 4 distinct subfamilies of chemokines they bind. Several chemokine receptors from different types, such as CCR1, CCR3, CCR2b, CCR8, CX3CR1 or CCR9 [45-47] can function as HIV coreceptors in cultured cells, but only two are known to play a role *in vivo*: CCR5 and CXCR4 [45].

CCR5 (CD195), a CCR chemokine receptor, is expressed on effector and memory T cells, natural killer cells, antigen presenting cells and microglia [48,49]. Its natural ligands include chemokines CCL3, CCL4 and CCL5, which have also the nomenclature MIP-1α (macrophage inflammatory protein), MIP-1β, and RANTES (regulated on activation normal T-cell expressed and secreted), respectively [50]. CCR5 is involved in inflammatory responses to infection, in the initiation and the amplification of the immune response [51-53].

HIV gp120 bind to the outer surface of CCR5, mainly by making contact with the N-terminus (Nt) and the second extracellular loop (ECL) of CCR5 [48,54,55]. Chemokines CCL3, CCL4 and CCL5 have been shown to be potent suppressors of HIV infection [56]. Also a 32 base pair deletion in the CCR5 gene, known as Δ32, was shown to protect against HIV infection [57] and slows disease progression. The mutant

allele codes for a dysfunctional truncated protein that is not expressed at the surface of the cell. Although homozygosis has been associated with an increased risk of symptomatic West Nile virus infection [58], this deletion seems not to have obvious effect on health.

CXCR4 (CD184) a CXC chemokine receptor called fusin, is an α-chemokine receptor specific for CXCL12, previously called stromal-derived-factor-1 (SDF-1α). It is expressed on a multitude of tissues and cell types and it has been shown to be involved in the homing and trafficking of leukocytes and haematopoietic progenitor cells, brain development, vascularisation, neonatal development, T-cell activation and migration at sites of inflammation and hematopoiesis [53,59]. Moreover, CXCR4 an its ligand has been described to be involved in different types of cancer, in the metastasis and angiogenesis [60].

Related to HIV infection, CXCL12 has been shown to have anti-HIV activity *in vitro* [61]. Several residues on CXCR4 transmembrane (TM) and ECL2 domains are involved in the interaction with HIV-1 gp120. Structure-function studies of CXCR4 have shown that there is a significant overlap between HIV-1 and chemokine CXCL12 functional sites [62], but particular mutations at the ECL2 on the extracellular regions of CXCR4 could reduce HIV-1 entry without impairing CXCL12 binding and signaling [63].

Envelope glycoproteins

The Env glycoprotein of HIV is composed by a surface subunit (gp120) and a transmembrane subunit (gp41) which form a trimeric functional unit consisting of three molecules of gp120 exposed on the virion surface and associated with three molecules of gp41 inserted into the viral lipid membrane [8,64,65]. It is synthesized from singly spliced mRNA as a 160 kD polyprotein precursor molecule (gp160) which is proteolytically processed by a host protease. First, it is synthesized in the endoplasmic reticulum (ER) and it is co-translationally glycosylated, then, gp160 monomers oligomerize, a process required for transport to the Golgi Complex. Once in the Golgi, Env undergoes modification of the N-glycosylation acquired at the ER. Also in the Golgi, gp160 undergoes proteolysis, generating the gp120 and gp41 subunits. Finally, the oligomeric, non-covalently associated gp120-gp41 complexes are transported to the cell surface, where they are incorporated into budding virions.

gp120

HIV gp120 is the surface subunit of Env and it is the main antigen promoting neutralizing antibodies [66]. It is highly variable between HIV isolates and this variability is not uniform within gp120. Assessment of the amino acid sequence of different HIV-1 strains led to the identification of five hypervariable domains (V1-V5) which are flanked by more conserved regions (C1-C5) [67]. Through gp120, there are series of highly conserved Cys residues, which are involved in nine intramolecular disulfide bonds crucial for the tertiary structure and function of Env [68]. The different regions are organized into an inner and an outer domain [37], connected by a four-stranded antiparallel β-sheet, called the bridging sheet. The four first variable regions form loops that remain exposed to the surface of the glycoprotein, whereas the most conserved regions are organized below them. At the inner part of gp120, there are the gp41 binding domains, composed by C1 and C5 regions. The HIV-1 gp120 glycoprotein is extensively glycosylated; in fact, approximately half the molecular mass of gp120 is composed of oligosaccharides [66,69]. These glycosylations are required for infectivity [70,71] and avoid the recognition of gp120 by the immune system [70].

Interactions between HIV and the cellular receptor and coreceptors, are mediated through specific domains of gp120. The gp120 determinants of CD4 binding map to C3 and C4 [64,72], whereas the V3 loop is the responsible for coreceptor interaction and it is an important determinant of the HIV-1 tropism [73]. To a less extent, V1/V2 and other regions have been described to have a role in coreceptor use.

The V3 loop is an approximately 35 amino acids long, glycosylated structure with a disulfide bond at the base and its sequence presents a high diversity between isolates [74,75]. The structure of V3 in the context of core gp120 bound to CD4, which has been described through crystal structures, reveals that V3 emanates from the gp120 outer domain and it acts as a molecular hook when it is going to interact with the coreceptor [76]. It can be subdivided into three structural regions (Fig. 7) [76]: a conserved base, which forms an integral portion of the core; a flexible stem, which extends away from the core; and a crown or tip, that includes the highly conserved GPGRAF sequence and that has a β-turn conformation [76-78]. Features of gp120 important for coreceptor binding have been mapped to the V3 tip, and the gp120 core around the bridging sheet (the V3 base, and neighboring residues) [79-81]. Analysis of these two regions indicates that they are conserved in both sequence and structure [76].

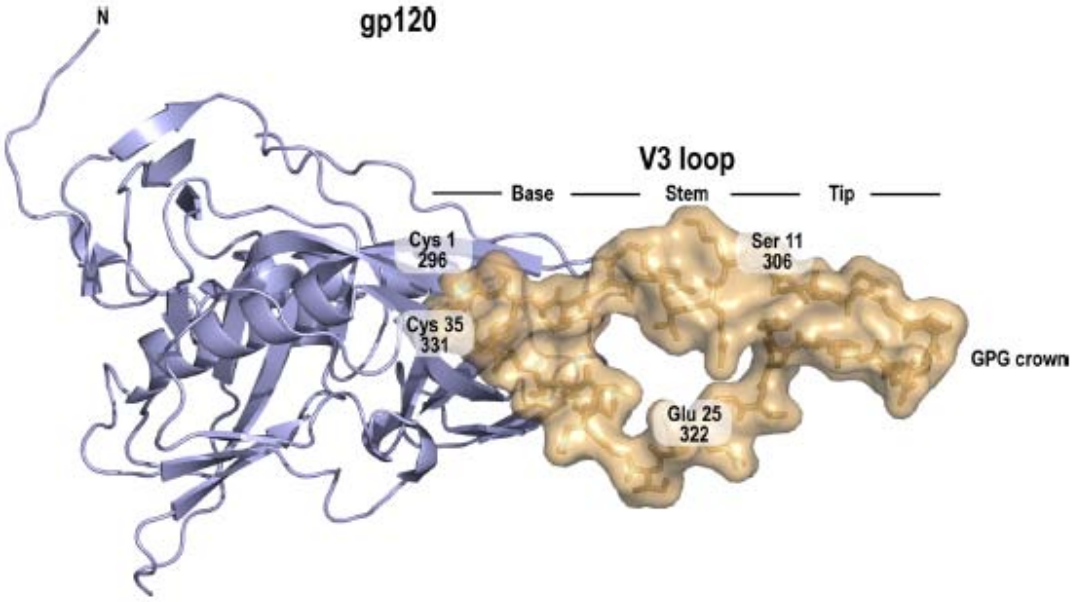


Figure 7. Crystal Structure of gp120 with the V3 . The V3 region is colored in Amber [78]

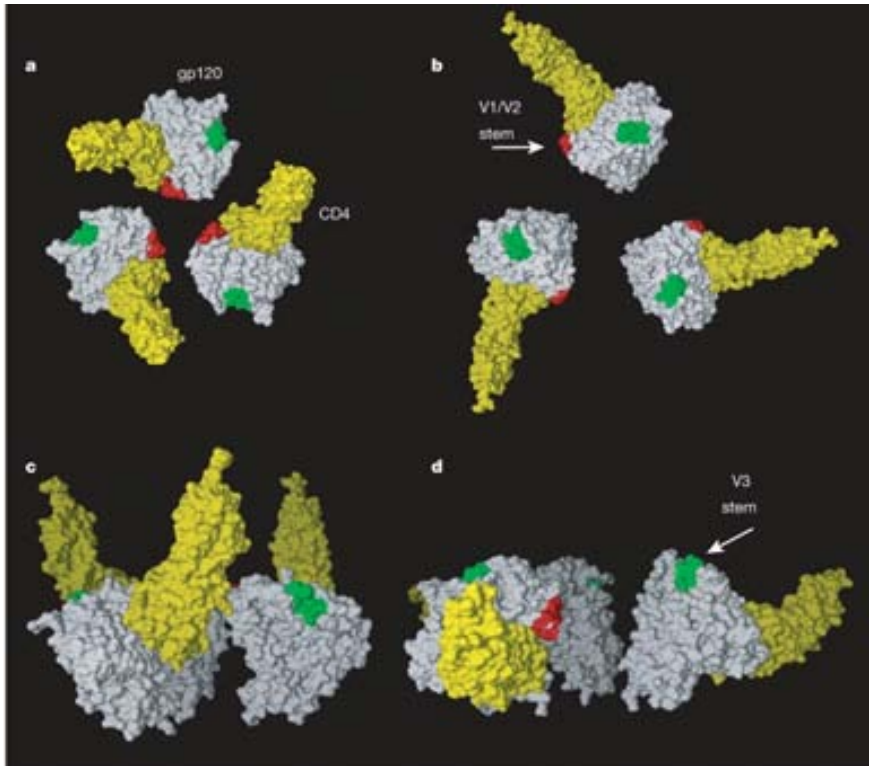


Figure 8. Model of the conformational change in the gp120 trimer induced by CD4 binding [65]**.** (A, C) gp120 unligated state, (B, D) CD4-bound state. (A, B) shown as top view and (C,D) shown as front view. The gp120 core, CD4, V1/V2 and V3 stems are shown in white, yellow, red and green, respectively.

Recent results using cryo-electron tomography combined with three-dimensional image classification and averaging, showed that upon gp120-CD4 binding, the Env trimer opens and makes way for exposure of the central gp41 stalk. The V3 region is realized from the lateral edge of the apex of the spike to directly point towards the target cell, while the V1/V2 regions as well as the CD4 binding sites move away from the centre of the spike (Fig. 8) [64,65,82]. These observations suggest that CD4 binding draws the spike closer to the target cell membrane.

gp41

HIV gp41 is the transmembrane subunit of Env and is critical for the fusion of the viral and cellular membranes. Structurally, gp41 consists of an extracellular, transmembrane (TM) and a cytoplasmic (CP) domain. The N-terminal fusogenic domain is at the external part of the virus and the C-terminal is in the inner side. Its extracellular domain contains four major functional regions. At the N-terminal region there is a hydrophobic, glycine-rich fusion peptide (FP) that will be anchored to the membrane of the host cell. Then, there are two regions with pseudo-repeated sequences of seven residues that form two α-helices: the N-terminal heptad repeat (NHR or HR1) and the C-terminal heptad repeat (CHR or HR2). Contiguously, there is a tryptophan-rich (TR) region.

In early studies, several peptides derived from the proteolysis of NHR (N-peptides) and CHR (C-peptides) were identified to have potent anti-HIV activity [83,84]. This led to a new class of antiretrovirals, which prevents membrane fusion, being the C-peptide T-20 (Fuzeon, enfuvirtide), the first anti-HIV agent targeting HIV entry to be approved for clinical use [85].

The finding of these anti-HIV peptides also provided important information to explore the structure of the gp41 molecule [86]. Some studies identified an alpha-helical domain within gp41 composed of a trimer of two interacting peptides. The crystal structure of this complex, composed of the peptides N36 and C34, is a six-helical bundle. Three N36 helices form an inner, parallel coiled-coil trimer, while three C34 helices pack in an oblique, antiparallel manner into highly conserved, hydrophobic grooves on the surface of this trimer [86,87]. Therefore, upon gp120 binding to CD4 and a coreceptor (CCR5 or CXCR4), gp41 changes its conformation to a fusion-active state characterized by the formation of a pre-hairpin intermediate, with NHR regions of gp41 forming a trimeric coiled coil structure that leads to insertion of the fusion peptide

into the target cell membrane. This allows the rearrangement of gp41 into a hairpin structure containing a thermodynamically stable six-helix bundle, by folding back the CHR helices over the hydrophobic grooves of the NHR coiled coil, that tethers together viral and cellular membranes allowing them to fuse [30].

HIV TROPISM

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) (Fig. 9) [88]. Due to the difficulty to distinguish between R5X4 variants and mixtures of R5 and X4 viruses, the term dual-mixed (D/M) is commonly used. Previously, X4 strains were known as SI (syncytium-formation), whereas R5 strains were denominated NSI (non-syncytium-formation) depending on their capacity to form syncytia in the CXCR4+ MT-2 cell cultures (MT-2 assay).

The coreceptor use is determined by the amino acid sequence of HIV gp120, in particular within V3 and V1/V2 loops, and less frequently within other regions [73]. For example, increase of positive net charge of V3 [81], the loss of N-glycosylation site [89] or the mutations S306R, D320R/Q, D324N [80,81] are associated to a coreceptor switch from CCR5 to CXCR4 usage. Other conserved changes in envelope during coreceptor switch have been described [90].

R5 viruses are characteristic of the asymptomatic stage of infection [91] and are selectively transmitted between individuals [92,93]. Over the course of the infection, the coreceptor usage of HIV changes from CCR5 to CXCR4 in 50% of infected individuals [94], although a higher percentage has been suggested on the basis of studies that used more sensitive detection techniques. In fact, increasing data seems to support the observation that the majority of patients carry a mixture of R5 and X4 using HIV strains [95-100]. The detection of X4 viruses has been associated with accelerated CD4+ T-cell decline and progression to AIDS [94,101], but the mechanisms leading to the emergence of X4 variants are not fully understood and many questions remain unanswered.

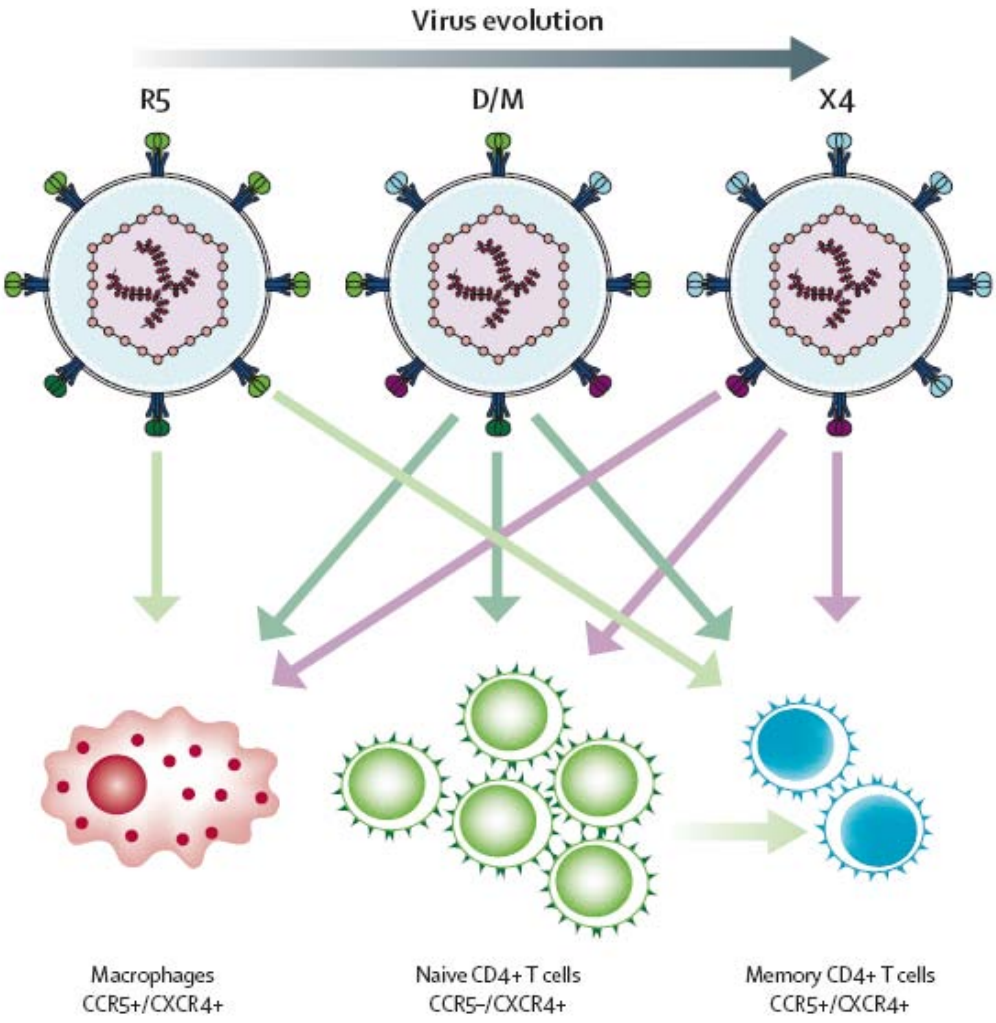


Figure 9. HIV-1 tropism [28]

The appearance of CXCR4 using viruses *in vivo* arises only after several years of infection. This is surprisingly slow given that changing only a few residues in gp120 is sufficient to convert an R5 virus into an R5X4 or X4 virus *in vitro* [80,81,102-104] and that such changes must be occurring constantly *in vivo* given the high replication rate of HIV and the error rate of the reverse transcription [105]. These observations imply that a selection pressure acts against the transition to CXCR4 usage *in vivo* and it is not clear if it has virological or immunological basis [106]. For instance, transitional R5 to X4 mutants have a diminished replication fitness, less-efficient coreceptor use, and unique mutational pathways, what could explain the long delay from primary infection until the emergence of CXCR4-using viruses [90,107]. On the other hand, an inhibitory role of antiviral immunity in HIV-1 coreceptor switch has been suggested [108-110].

Alternatively, CCR5 and CXCR4 are preferentially expressed in memory and naïve T cells, respectively, and a change in the relative abundance of memory to naïve T cells may favor the emergence of X4 viruses later during the course of disease [111].

It is unclear, if the emergence of X4 strains is a cause or consequence of the progression to AIDS. There are descriptions of *in vitro* cytopathicity of X4 viruses [112,113]. Also recent studies show that X4 strains appear to emerge well before AIDS onset, and therefore, are likely to be etiologically linked to CD4+ T cell decline and progression to AIDS [114]. The emergence of X4 strains reflects an increased risk of HIV-1 disease progression. Then, it is suggested that detection of X4 strains, should be considered as a potential biomarker to guide clinical management throughout HIV infection [115].

ANTI-HIV TREATMENT

Since 1996 antiviral therapy consisted in the so called highly active antiretroviral therapy (HAART) which consists in the combination of three or more anti-HIV compounds targeting at different levels the HIV replication cycle. For a long period of time the drugs employed in HAART were viral enzyme inhibitors, such as the reverse transcriptase (RT) inhibitors (nucleoside analogues or non-nucleoside RT inhibitors, NRTI and NNRTI respectively) or protease inhibitors (PI).

The current antiretroviral treatment has reduced morbidity and mortality in HIV-1 infected individuals. However, it is often limited by the emergence of drug-resistant HIV-1 strains. HIV infection is characterized by a high replication rate [105] and together with the extremely error-prone process of reverse transcription, numerous mutations may occur during this step. Such mutations generate a genetically diverse population within an infected person that may lead to the selection of viruses better replicating in a drug-containing environment. For this reason when treatment is not able to totally inhibit viral replication, a resistant viral population may emerge. Moreover, all the anti-HIV compounds present long-term toxicity and adverse effects [116], lowering the adherence to treatment, what leads to a suboptimal concentration of the compounds and subsequent viral failure with development of resistances. The drug-resistance emergence together with the latency of HIV [117] and the presence of viral reservoirs

[117] where the drugs cannot achieve the optimal concentrations, makes the current treatments unable to eradicate the virus from infected individuals.

Recently a large number of inhibitors targeting different steps of HIV cycle have been developed, mainly targeting HIV entry (explained below). The last inhibitor to be approved for clinical treatment by the Food and Drug Administration (FDA) in October 2007 was the first integrase inhibitor raltegravir [118].

Entry inhibitors

HIV-1 entry into host cells is an essential step that offers several potential new targets for antiviral agents [28]. Up to the present, two entry inhibitors have been approved for HIV treatment, whereas some others are in clinical trials (Table 1). Enfuvirtide (T-20), a fusion inhibitor, was the first entry inhibitor to be approved by the FDA and in April 2007 a CCR5 antagonist, maraviroc, was approved for treatment of drug-experienced patients.

Table 1. Entry inhibitors in clinical trials.

Compound	Target	Status (last update)	Developer
TNX-355	CD4	Phase II	Tanox
Sifurvitide	gp41	Phase I	Fusogen
AK602	CCR5	Phase I	Kumamoto University
PRO140	CCR5	Phase II	Progenics
Vicriviroc (SCH-D)	CCR5	Phase III	Schering-Plough
INCB9471	CCR5	Phase II	Incyte Corporation
TAK-652/TBR-552	CCR5	Phase I	Takeda
HGS004	CCR5	Phase I	Human Genome Sciences
SP01A	cholesterol	Phase II/III	Samaritan Pharma

Attachment Inhibitors

There are several compounds inhibiting the attachment of HIV particles to the cell surface. Polyanions are molecules of different structures and sizes, such as dextran sulfate (DS) and heparan sulfate (HS), that have been shown to block HIV-1 replication through their capacity to block the attachment of free virions to CD4+ cells. However, some results suggest that HS may bind to the HIV coreceptor binding site of gp120 and block virus–coreceptor interactions. Thus, some polyanions may have a secondary mode of action at a later stage than virus–cell attachment [119]. Another well-known polyanion is zintevir (AR177 or T30177), which has 17 nucleotides and that first, it was believed to inhibit the HIV integrase enzyme, but later it was observed to inhibit at the gp120 level [120].

Binding inhibitors

The mechanism of action of some binding inhibitors, such PRO542, TNX355 and BMS488043, is the inhibition of the gp120/CD4 interaction.

First efforts to block HIV entry implicated the use of soluble CD4 (sCD4) [121]. It binds to the gp120 from the HIV particles, inhibiting its binding to the cellular CD4 receptor. However, in clinical trials it did not have good results due to the lost of anti-HIV activity against clinical isolates compared to the laboratory adapted strains.

Later, some mimics of CD4 receptor were developed, being CD4M33 the most promising candidate [122]. It is a 27-amino acid CD4 mimic that was designed using structural information on a CD4-gp120-17b antibody complex. CD4M33 presents optimal interactions with gp120 and binds to viral particles and diverse HIV-1 envelopes with CD4-like affinity, including primary patient isolates that are generally resistant to inhibition by sCD4. Furthermore, CD4M33 possesses functional properties of CD4, including the ability to unmask conserved neutralization epitopes of gp120 that are cryptic on the unbound glycoprotein. CD4M33 is a prototype of inhibitors of HIV-1 entry and, in complex with envelope proteins, it is a potential component of vaccine formulations, or a molecular target in phage display technology to develop broad-spectrum neutralizing antibodies [122,123].

PRO542 (CD4-immunoglobulin G2) [124] from Progenics is a tetravalent CD4-immunoglobulin fusion protein comprising human IgG2 in which the Fv portions of

both heavy and light chains have been replaced by the D1 and D2 domains of human CD4. It broadly neutralizes primary HIV-1 isolates, by binding to the viral surface glycoprotein gp120 and blocking attachment and entry of the HIV particles. In clinical trials it proved to have anti-HIV activity and to be well tolerated [125]. However, PRO-542 is no longer under active development for the treatment of HIV infection.

BMS-378806 (Bristol-Myers Squibb) is a small molecule HIV-1 inhibitor that blocks viral entrance to cells. The compound exhibits potent inhibitory activity against a panel of R5, X4, and R5/X4 HIV-1 laboratory and clinical isolates of the B subtype in culture assays [126]. The median 50% effective concentration (EC_{50}) calculated was 0.04 μM. Mechanism of action studies demonstrated that BMS-378806 binds to gp120 and inhibits the interactions of the HIV-1 envelope protein with cellular CD4 receptors. BMS-378806 displays favorable pharmacological traits, good oral bioavailability in animal species, and a clean safety profile in initial animal toxicology studies [126]. BMS-488043 like its predecessor BMS-378806, targets the initial gp120-CD4 interaction of viral entry and shows potent antiviral activity against R5 and X4 tropic laboratory and clinical isolates [126-128]. With improved pharmacokinetic properties, BMS-488043 demonstrated antiviral efficacy and a favorable safety profile in HIV-infected subjects [129]. Nevertheless, in August 2004, development of this drug was halted by its manufacturer and they will continue studying other drugs that act very much like BMS-488043, in order to maximize their effectiveness.

The monoclonal antibody (mAb) TNX-355 (formerly known as Hu5A8) is a humanized IgG4 mAb that inhibits HIV-1 entry by an unique mechanism [130-132]. This antibody, like its murine progenitor (Mu5A8), binds to extracellular domain 2 of rhesus and human CD4, thereby preventing postbinding entry of the virus into CD4+ cells. Humanized and murine mAbs inhibit the *in vitro* infectivity of diverse primary isolates of HIV-1 with EC_{50} values of 0.0004 - 0.152 mg/ml [130,131]. The antibody binding site on CD4 is distinct from the site required for the binding of HIV-1 envelope gp120. Also, unlike anti-CD4 antibodies that target domain D1, Mu5A8 and TNX-355 do not interfere with immunological functions that involve antigen presentation. Therapeutic immunoglobulin molecules typically have long half-lifes, which permits relatively infrequent dosing. Results from two clinical trials demonstrate the feasibility of inhibiting HIV-1 *in vivo* by a CD4-specific mAb [132,133], however, a more definitive assessment of the safety and therapeutic potential of TNX-355 is needed.

Before the drug can move into phase III clinical trials, the FDA has requested additional early-stage phase II studies to determine the correct dose of the drug.

CCR5 coreceptor inhibitors

Individuals homozygous for the Δ32 mutation of CCR5 (explained in *Coreceptors* section) are almost completely resistant to HIV infection and this mutation seems not to have obvious health consequences. These observations suggested that small molecules that could prevent HIV interaction with CCR5 could form a promising class of antiretroviral drugs and several compounds targeting CCR5 were developed.

After the discovery of CCR5 as an HIV coreceptor and that its natural ligands CCL3, CCL4 and CCL5 had an anti-HIV effect by internalizing the chemokine receptor, various modified chemokines were designed. (AOP)-RANTES, (NNY)-RANTES and (PSC)-RANTES induce the internalization of CCR5 and inhibit the recycle to the surface of the cells [134,135].

The more developed CCR5 targeting compounds are the small-molecule CCR5 antagonists. These agents have shown significant potency in cell culture across diverse strains and clinical isolates with nanomolar EC_{50} [136-140] and have shown short-term antiretroviral activity and efficacy in clinical trials [141-144]. Apparently they inhibit HIV replication by binding to a pocket within the transmembrane helices of the receptor, which alter extracellular CCR5 conformation [145]. The names of agents that have progressed to clinical trials end in the suffix-viroc (e.g., aplaviroc) to denote their action of viral receptor occupancy.

Takeda Pharmaceutical Company Ltd. (Osaka, Japan) was the first one to describe a non-peptidic small-molecule able to inhibit R5 viruses by targeting CCR5. This CCR5 antagonist was TAK-779 [136] and it was a highly potent inhibitor *in vitro* at nanomolar concentrations. However, based on TAK-779, TAK-220 and TAK-652 were developed in order to improve bioavailability. Both compounds showed a potent HIV inhibition at nanomolar concentrations (EC_{50} 0.5-1.7 nM and 0.5-2.4 nM) in PBMC and oral bioavailability [146,147]. A phase I study was completed for TAK-652 showing favorable pharmacokinetics and safety data [146]. Tobira Therapeutics (Princeton, NJ, USA) is continuing TAK-220 and TAK-652 development through phase I clinical trials under the names of TBR-220 and TBR-652, respectively.

After the description of TAK-779, Schering-Plough (Kenilworth, NJ, USA) developed SCH-C or SCH351125 [148]. It demonstrated a broad and potent antiviral activity against R5 viruses [148] and showed *in vivo* efficacy in clinical trials (0.5-1log decrease in VL in 10/12 subjects at a dose of 25 mg twice/day), providing proof of concept for CCR5 antagonists as valid inhibitors *in vivo*. Nevertheless, it caused prolongation of the cardiac QT interval. This resulted in an improved derivative, SCH417690 or SCH-D, later named vicriviroc, that continued further development. It had 2- to 40-fold more potency than SCH-C, inhibiting a broad panel of R5 primary isolates *in vitro* at nanomolar concentrations and it had an improved safety profile and an excellent oral bioavailability [139]. Vicriviroc as a monotherapy in treatment-naïve patients showed reductions of 1.5 and 1.6 log in VL at doses of 25 mg and 50 mg twice/day [149]. In a phase II study, treatment-naive patients (n=92) with R5 viruses received vicriviroc monotherapy at 25, 50 or 75 mg/day for 2 weeks, and then combivir (AZT/3TC) was added (placebo consisted in efavirenz plus combivir). Treatment with vicriviroc led to a 0.9-1.3 log greater decrease in VL than in patients receiving placebo [150]. In the ACTG A5211 study, in treatment-experienced patients (n=118) decreases in mean VL were greater in the vicriviroc groups compared to the placebo ones: 1.51, 1.86 and 1.68 log (for 5 mg, 10 mg and 15 mg doses, respectively) versus 0.29 for placebo [142]. Vicriviroc is currently at phase III trials.

Another CCR5 antagonist, aplaviroc (APL/AK602/ONO4128/GSK-873140), was initially designed by the Japanese company Ono Pharmaceuticals (Osaka, Japan) [151], but was further developed by GlaxoSmithKline (Brentford, Middlesex, UK). Aplaviroc showed high antiviral potency and very slow receptor dissociation [151,152]. In an early phase study, it proved to reduce the mean VL by a 1.66 log after 10 days with a maximum concentration of 600 mg twice/day [153]. However, later clinical trials were stopped at phase IIb/III because of severe hepatotoxicity [154].

Maraviroc (MVC/UK-427,857) [137] was developed by Pfizer Inc. (kent, UK). This small molecule CCR5 antagonist is currently approved for the treatment of drug-experienced HIV+ patients. MVC demonstrated excellent antiviral activity against R5 viruses *in vitro* [137] and in clinical trials it demonstrated to be a valuable treatment option for patients harboring R5 HIV viruses. Two phase II studies addressed the efficacy of short-term (10 days) MVC monotherapy in 82 R5 tropic HIV patients. With one exception, all patients had a decrease in VL > 1.0 log copies/ml [155]. In the phase

II/III MOTIVATE 1 and 2 studies (n=1049) treatment with maraviroc plus optimized background therapy (OBT) was associated with significantly greater virologic and immunologic efficacy at 48 weeks and had a similar safety profile, as compared with placebo plus OBT groups [156]. More patients receiving maraviroc once or twice daily had HIV-1 RNA levels of less than 50 copies/ml (42% and 47%, respectively, vs. 16% in the placebo group in MOTIVATE 1; 45% in both maraviroc groups vs. 18% in MOTIVATE 2). The change from baseline in CD4 counts was also greater with maraviroc once or twice daily than with placebo [156].

INCB9471 [157] of Incyte Corporation (Wilmington, Delaware, USA) is being developed as a once-daily treatment for HIV, it has been studied at three doses in a phase IIa 14-day trial in which the compound provided potent and prolonged antiviral effects in HIV patients with R5-tropic viruses. It was well-tolerated at all three doses [158]. The compound is currently being evaluated in several drug interaction trials to support the initiation of phase IIb six-month trials in treatment-experienced HIV patients.

Several monoclonal antibodies targeting CCR5 have also been described, but only two have been tested in clinical studies: CCR5mAb004/HGS004 of Human Genome Sciences (Rockville, MD, USA) and PRO140 of Progenics Pharmaceuticals (Tarrytown, NY, USA).

HGS004 is a fully human mAb that specifically binds to the ECL2 of CCR5 and inhibits R5 HIV-1 entry and chemokine signaling with similar efficiencies [159]. It demonstrated to have anti-R5 HIV effects *in vivo* and to be safe and well tolerated in phase I trials [160] .

PRO140 is an humanized mouse-derived (PA14) mAb, which binds to an epitope spanning ECL2 and Nt of CCR5 [161]. The mouse mAb PA14 and PRO140 have shown a broad and potent activity against R5 viruses in preclinical studies [161]. In a phase I study, PRO140 proved to have a potent, prolonged, and dose-dependent antiviral activity with minimal toxicity in patients harboring R5 HIV-1 viruses [162]. PRO140 is considered as a fast-track product by the US Food and Drug Administration.

CCR5 mAb and small-molecule CCR5 antagonists have different binding sites and modes of action. PRO140 binds to the Nt and ECL2 of CCR5 and acts as a direct competitive inhibitor of HIV binding. Conversely, the small-molecule antagonists bind

in a hydrophobic pocket of CCR5 formed by transmembrane helices and seems to act through allosteric effects. For this reason viruses resistant to CCR5 antagonists could maintain sensitivity to PRO140 [163-165] and *in vitro* assays suggest that they have synergistic effects [166,167].

CXCR4 coreceptor inhibitors

Bicyclams were the first class of CXCR4 agents described to block HIV replication [140,168-170], but a number of different agents including synthetic peptides, peptoids, arginine conjugates, polyanions or CXCL12 derived-peptides have been developed since then.

The bicyclam AMD3100 (Mozobil™, plerixafor), previously called JM3100 and SID791, was the first non-peptide, small-molecule CXCR4 inhibitor described [140,168-171]. It inhibited HIV-1 and HIV-2 strains with an EC_{50} of 1-10 nM and also proved to be potent against R5X4 strains in PBMC. It showed specific interaction with CXCR4 and blockage of calcium signaling, chemotaxis and CXCR4 down-regulation induced by CXCL12. Clinical studies with AMD3100 in HIV-infected patients provided evidence for CXCR4 antagonism to have a clinical anti-HIV effect [172]. Nevertheless these studies had to be stopped due to undesired cardiac effects. Further development of this compound gave the non-cyclam inhibitor AMD070 or AMD11070 (AnorMED), which presented a reduction in both molecular size and charge and made it the first orally bioavailable small-molecule CXCR4 inhibitor. AMD070 potently inhibited X4 viruses with an EC_{50} of 1-15 nM in T cell lines and PBMC. In a phase Ib/IIa, it demonstrated to be active in HIV-infected patients harboring CXCR4-using viruses [173]. It was evaluated in a pilot monotherapy study with patients harboring X4 or R5/X4 viruses [173], but was suspended after the observation of liver histology changes and liver and retinal toxicity in animal research studies. However, a greater than 1 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*.

Other compounds have been designed based on AMD3100. AMD3465 [174] is a cyclam that fully conserves all the biological properties of AMD3100, whereas it lacks some structural constrains such as the high positive charge that gives poor oral bioavailability. Compared to the bicyclam AMD3100, AMD3465 was even 10-fold

more effective as a CXCR4 antagonist. Recently, another group, discovered novel potent CXCR4 inhibitors from a library of non-cyclam polynitrogenated compounds that preserved the main features of AMD3100 [175].

Cyclam CXCR4 inhibitors, similarly to small-molecule CCR5 antagonists, are proposed to bind to the TM regions of the receptor, which consequently causes a conformational change leading to the disruption of receptor function and avoiding recognition by gp120 from HIV particles. However, these CXCR4 inhibitors have potential for multiple ionic interactions whereas the CCR5 inhibitors form mainly hydrophobic interactions with CCR5 [176].

Another of the first CXCR4 antagonists described was polyphemusin II, a natural 18-amino acid peptide isolated from the American horseshoe crab [177]. Based in this peptide, T22 was developed. It is a synthetic cationic peptide that inhibits X4 strains by binding to the Nt and two ECL of CXCR4 [177]. Later two analogues were developed, T134 and T140, which showed a stronger potency in inhibiting X4 entry [178].

A polypeptide of nine Arg, ALX40-4C [169], was initially characterized as an inhibitor of Tat binding to the trans-activation-response-element (TAR). However, it was demonstrated that it inhibited entry of X4 viruses, but not R5 viruses. In particular, it inhibited NL4-3 at nanomolar concentrations with a mean EC_{50} of 3 nM. ALX40-4C was well tolerated in phase I/II clinical trials in humans, but no significant reductions in viral load were noted [179].

Some peptoids such as CGP64222 [180] were also described as CXCR4 inhibitors. CGP64222 is a basic peptoid oligomer of nine residues that inhibited both Tat/TAR binding and the gp120 binding to CXCR4, what was demonstrated by the fact that it had no activity against HIV-1 strains resistant to bicyclams or R5 strains and blocked CXCL12-induced calcium signaling.

KRH-1636 (Kureha Chemical Industries) is another small-molecule CXCR4 antagonist that has a potent anti-HIV activity against X4 strains, including clinical isolates both *in vitro* and in a mouse model [181].

Finally, after having found that many residues throughout CXCR4 TM and ECL2 domains were specifically involved in interaction with HIV-1 gp120, and that most of these sites did not play a role in either CXCL12 binding or signaling, several

synthetic chemokines were designed [182]. These chemokines are termed synthetically and modularly modified (SMM) chemokines and are derived from the native sequence of CXCL12. They can inhibit selectively the entry of X4 viruses without affecting the CXCL12 signaling [182].

Dual CCR5 and CXCR4 inhibitors

The low-molecular weight compound AMD3451 [183] is a cyclam analog that has been described to be the first anti-HIV compound targeting both CCR5 and CXCR4 coreceptors. It showed anti-HIV activity against R5, R5X4 and X4 strains (EC_{50} of 1.2 to 26.5 μM) in T cell lines and in PMBC. Although it proved to act as an specific antagonist of CCR5 and CXCR4 receptors, the precise interaction sites with these receptors have not been described yet.

gp41-mediated fusion inhibitors

HIV envelope glycoprotein transmembrane subunit gp41 plays a critical role in the fusion between viral and target cell membranes. Gp41 organization in its fusogenic active conformation originated the design of HR derived peptides [83,84]. When anti-HIV activity of the derived peptides from the N-terminal or HR1 (N-peptides) or from the C-terminal or HR2 (C-peptides) was analyzed, it was observed that the C-peptides were more potent. Within this group there are the DP178 or T-20, T1249, T649 and C34 peptides.

T-20 (Enfuvirtide, ENF, Fuzeon™ by Trimeris/Roche Inc) [85] comes from a gp41 sequence of HIV-1 subtype B. A relatively long peptide such as T-20 suffers from several limitations including lack of oral bioavailability and high cost of production.

A second generation of fusion inhibitor peptides, derived from the HR2 region of TM gp41, (Trimeris/Roche Inc.) are being evaluated in preclinical studies [184]. They presented a better antiviral activity against wild-type (wt) viruses than T-20 and were also active against T-20-resistant strains. In addition, TR-291144 and TR-290999 showed an improved bioavailability that allow them to be administrated once weekly instead of twice daily.

Recently, other fusion inhibitors peptides have been described [185-188] trying to improve the potency, solubility and stability. One of them, VIRIP, is a 20

residue peptide extracted from human hemofiltrate. It blocked HIV by interacting with the gp41 fusion peptide and its optimization (VIR-353) increased its antiretroviral potency by two orders of magnitude [189]. Sourprisigly, the authors who discovered VIRIP suggested that it was not posible to develop a resistant virus to this class of inhibitor. Another interesting peptide is sifuvirtide [190], a 36 residue peptide that was designed based on the three-dimesional structure of the gp41 fusogenic core conformation. It showed to be active against the T-20-resistant strains.

Lipid rafts and actin cytoskeleton targeting agents

Statins are effective cholesterol-lowering agents. They inhibit the 3-hydroxy-3-methylglutaryl coenzme A (HMG-CoA) reductase, which produces mevalonic acid, a precursor for cholesterol biosynthesis and generation of isoprenoids [191]. The inhibition of this enzyme in the liver stimulates LDL receptors, resulting in an increased clearance of low-density lipoprotein (LDL) from the bloodstream and a decrease in blood cholesterol levels. The statins are divided into two groups: fermentation-derived (lovastatin, mevastatin, simvastatin and pravastatin) and synthetic (fluvastatin, atorvastatin, cerivastatin and rosuvastatin). They are used to treat hypercholesterolemia and they are extensively used in medical practice. In addition, statins are currently being used in HIV+ individuals in order to reduce the hyperlipidemia that is frequently induced by antiretroviral treatment [192] and that increases the risk of cardiovascular disease [193-195].

Recent studies suggested that statins had direct anti-HIV effects *in vitro* [32,196]. Also, in a short short-term study, lovastatin showed to have an effect in 6 HIV+ patients treated with this statin [197].

One possible mode of action of statins would be by lowering the cholesterol levels. Both CD4 and chemokine coreceptors for HIV are found disproportionately in lipid rafts and removing cholesterol from virions, producer cells, or target cells could decrease the infectivity of HIV. Another mechanism proposed to explain its anti-HIV effects is that statins, by targeting Rho GTPases, affect the actin cytoskeleton rearrangement [197] necessary for virus entry or budding from target cells. Alternatively, it has been proposed that statins suppress intercellular cell adhesion molecule 1 (ICAM-1)–leukocyte function antigen 1 (LFA-1) interactions that are

required for viral entry [198]. Statins have also been proposed as agents that modulate the immune system [199,200] and have anti-inflammatory properties [201], by inhibiting CCL3 [202] or other cytokines and chemokines, downregulating MHC II [200] or inhibiting CD40 expression [203]. This modulation of the immune response also could have a relevant role in the HIV infection.

Another compound targeting lipid rafts is SP01A (procaine hydrochloride), from Samaritan Pharmaceuticals. It reduces intracellular cholesterol and corticosteroid biosynthesis by reducing HMG-CoA mRNA levels [204]. SP01A is an oral HIV entry inhibitor that has demonstrated significant efficacy in preventing HIV replication *in vivo*. Samaritan Pharmaceuticals has performed pivotal phase IIb/IIIa clinical trials for HIV patients failing triple therapy due to viral resistance and SP01A is continuing further development (http://www.samaritanpharma.com/aids_hiv_program_sp-01a.asp).

HIV-1 resistance to CCR5 inhibitors

A potential problem of this approach is that under CCR5 selective pressure it is possible that HIV evolves to use CXCR4, by selecting for populations with *de novo* ability to use CXCR4 or that were already present in the patient as minor populations [205]. The main concern about X4 or R5X4 emergence is the unknown consequences it could have in CD4+ T cell decline or in the course of infection. Nevertheless, *in vitro* resistance to CCR5-targeting drugs has often been associated with genotypic and phenotypic changes that appear not to promote the emergence of CXCR4-using strains, despite the requirement of few amino acid changes for R5 viruses to switch [164,165,206-208]. Resistance to CCR5 antagonists seems to involve the use of CCR5 in complex with the inhibitor, but the resistant virus still can use the inhibitor-free CCR5 [206,209]. This mechanism, termed noncompetitive or allosteric, confers a diminished “plateau” or maximum percent inhibition value at a saturating inhibitor concentration. Contrary to the competitive resistance, the noncompetitive one does not involve a change in the $EC_{50.}$ The resistance described in studies with the vircriviroc precursor AD101 [164,207], maraviroc [206] and TAK-652 [208] involved 2 to 4 mutations in the V3 loop, although development of SCH-D resistance lead to mutations within gp120 and not in V3 region [165]. Also, for vicriviroc resistance, mutations in gp120 were

described and included mutations in the V3 loop, although they were dependent on the HIV-1 backbone in which they were expressed [210].

Little information on CCR5 antagonists resistance is available from *in vivo* use of these inhibitors, but the emergence of CXCR4-using viruses appears to be the most common cause of treatment failure. In phase III clinical trials of maraviroc (MOTIVATE 1 and 2), among patients that failed treatment, the emergence of CXCR4-using viruses was more common among maraviroc than in placebo-receiving individuals. In fact, X4 or D/M viruses were detected in 51% (35/68) and 63% (41/65) of the failed maraviroc treated patients with one or two doses of maraviroc per day respectively, compared to only 6 % (6/95) who failed placebo [211]. Similarly, in MERIT phase 3 trial in treatment-naïve individuals, X4 or D/M viruses emerged in 31% (10/35) of patients receiving maraviroc as a first-line regimen at the time of virologic failure [212]. Concerning the vicriviroc ACTG A5211 phase IIb trial, in a 35% (9/26) of treatment-experienced patients that failed vicriviroc therapy, the cause was the emergence of CXCR4-using variants [142].

The restriction of CCR5 antagonists use to patients only with R5 viruses makes determination of the coreceptor tropism a requisite for initiation of a CCR5 inhibitor treatment. However, as explained in next section, the Trofile screening test used to determine the coreceptor use in these clinical trials is not very sensitive and the detection of CXCR4-using viruses at treatment failure seems to be mainly due to the selection of pre-existing X4 or R5X4 viruses [205,213].

Tropism assays

Several methods have been developed to determine the tropism of HIV populations. Those methods include traditional phenotypic assays (determining replication of clinical isolates in MT-2 cells or U87 cells expressing one or the other coreceptor), phenotypic assays with env-recombinant viruses and bioinformatics-driven predictor assays based on the *env* sequence.

Phenotypic recombinant virus assays are based on the recombination of the *env* gene amplified from patient samples into a reporter HIV-1 backbone. There are several available: Trofile (Monogram Biosciences), Phenoscript (VIRalliance), XtrackC/ PhenX-R (inPheno) and a platform developed by Virco [98,214-217]. However, PhenoSence HIV Coreceptor Tropism (Trofile) is currently the only tropism assay that

has been clinically validated for determination of coreceptor use. It has been used in the clinical testing of maraviroc and vicriviroc. However, minimal VL of 1000 RNA copies/ml is required for adequate sensitivity (94%-95% amplification success rate). Therefore, the limit of detection of minor HIV strains in this assay is 5-10% or 10-20% [218,219]. An enhanced sensitivity version of Trofile (Trofile ES) has been developed and it showed to have a 10-100-fold improved ability to detect low levels of CXCR4-using variants compared with the standard Trofile [220].

The genotypic assays are based on the observation that most of the determinants for coreceptor use map to the V3 loop of gp120. Several bioinformatic tools have been developed by prediction algorithms, some of which are available on the internet such as C4.5, PART, SVM, Charge Rule, PSSMsinsi, PSSMx4r5 and geno2pheno [81,99,221-224]. The predictive value for X4 viruses of most of these algorithms is high, but the problem is that there is a lack of sensitivity to detect minor species when sequencing the patient viral population [99]. Nevertheless, they have a great potential because they can be faster and cheaper than phenotypic assays for tropism routine determination, but good genotype-phenotype correlations are still required before they can be used.

OBJECTIVES

HIV treatment is limited by multidrug resistance and intolerance, therefore any new compound is of great interest. However, their anti-HIV profile, benefits, risks and consequences must be carefully studied. In the studies presented herein, we tried to asses the anti-HIV activity of statins as antiretroviral agents, determine the role of coreceptor antagonists on the emergence of CXCR4-using strains and characterize a novel CXCR4 inhibitor.

Our specific objectives were:

Characterization of statins as potential antiretroviral agents:

- To determine the potency, efficacy and cytotoxicity of statins as anti-HIV compounds and study its mechanism of action.
- To analyse the anti-HIV effect of simvastatin on the viral load and CD4+ T cells counts in a pilot study conducted in the *Unitat de SIDA de l'Hospital Universitari Germans Trias i Pujol.*

Determination of the role of HIV coreceptor antagonists in coreceptor switch:

- To develop an assay that would allow the study of the R5 HIV-1 evolution in tropism.
- To study the effect of CCR5 and CXCR4 targeting agents on the evolution of different HIV-1 strains with respect to their coreceptor use.

Characterization of the novel CXCR4 antagonist POL3026 as an antiviral agent:

- To assess the anti-HIV potency and specificity of POL3026
- To determine the mechanism of action of POL3026
- To evaluate the consequences of POL3026 as a CXCR4 antagonist on coreceptor switch

MATERIALS AND METHODS

CELLS

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 [225].

Primary cells

Peripheral blood mononuclear cells (PBMC) were purified from healthy donors; they 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 were activated with PHA and interleukin-2 (IL-2) for 72 h. In some assays (*HIV-1 Escape to CCR5 coreceptor Antagonism through Selection of CXCR4-using variants in vitro* and *Anti-HIV Activity and Resistance Profile of the CXCR4 antagonist POL3026* sections), PBMC from 6 different donors 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 in liquid nitrogen until need. Monocytes and CD4+ T cells were purified from PBMC by negative selection (StemCell, Vancouver, Canada) as indicated by the manufacturer. Purity of preparations was assessed by staining with CD3-FITC/CD14-APC for monocytes and TriTEST reagent (CD4-FITC/CD8-PE/CD3-PerCP) for CD4+ T cells (BD Biosciences, Madrid, Spain) and analyzed by flow cytometry (LSRII. BD Madrid, Spain). Purity of monocytes and CD4+ T cell preparations was >85% and >95%, respectively. Macrophages were obtained by culturing 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.

REAGENTS

Chromatographically and spectroscopically pure atorvastatin lactone, simvastatin, their sodium and calcium salts and fluvastatin calcium salt were prepared at the *Departament de Quimica Organica, Universitat de Barcelona* (Barcelona, Spain). Samples of the natural product lovastatin (mevinolin) and of sodium fluvastatin were a gift of the pharmaceutical company Fyse-Ercros (Aranjuez, Spain). Additional lovastatin samples were purchased from Sigma-Aldrich (St. Louis, USA). The stabilities and purities of the stock dimethyl sulfoxide (DMSO) solutions of statins were checked for two representative samples, by solving 20 mg of calcium atorvastatin and of simvastatin in DMSO-d_6 (0.6 ml) and registering the 400 MHz ^{1}H NMR spectra once a day during one week; no changes (dehydration, lactonization/ring-opening equilibria, or other reactions) were observed.

The synthesis, purification, and chemical characterization of the CXCR4 antagonist POL3026 (Fig 1.) was performed as described before [226] by Polyphor biotech company (Allschwil, Switzerland).

The chemokines CXCL12 (natural ligand of CXCR4) and the natural ligands of CCR5 CCL3, CCL4 and CCL5 were purchased from Peprotech (London, UK). The RT inhibitor 3-Azido-3-deoxythymidine (zidovudine, AZT) was 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 [48,140,169,177,227]. The CCR5 antagonist TAK-779, the RT inhibitors efavirenz, nevirapine and lamivudine (3TC) were received from the NIH AIDS Research and Reference Reagent Program and the monoclonal antibody anti-CCR5 PRO140 from Progenics Inc. The anti-CCR5 monoclonal antibody, clone 2D7, was purchased at Becton Dickinson (Madrid, Spain). The fusion inhibitor T-20 (enfuvirtide) 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 MRC Centre for AIDS Reagents (London, UK). HIV strains from subtypes B (92BR014), A (92UG029), A (39RW029), D (92UG021), D (92UG024), F (93BR020) and O (BCF06) were obtained also 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 [73,227]. The IRLL98 HIV-1 strain [228] 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 CXCL12 resistant strain were derived after sequential passage of the NL4-3 virus in the presence of increasing concentrations of AMD3100 or CXCL12 in MT-4 cells [229].

The X4 HIV-1 clinical isolate CI-1-SI, the R5 clinical isolates CI2, CI3 and CI4 and the dualtropic CI6, CI7 and CI8 were obtained by coculturing PBMC from HIV-1-infected patients with stimulated PBMC from healthy donors. CI5 correspond to the HIV-1 168.1, which is a R5 molecular clone virus isolate obtained from a HIV-infected patient [80].

ANTIVIRAL AND CYTOTOXICITY ASSAYS

Anti-HIV assays in MT-4 cells

Anti-HIV activity and cytotoxicity measurements in MT-4 cells were based on viability of cells that had been infected or mock-infected with HIV-1 at a multiplicity of infection (MOI) of 0.003 and exposed to various concentrations of the test compound. After 5 days of infection, the number of viable infected and mock-infected cells was quantified by a tetrazolium-based colorimetric method (MTT method) as described [227,230]. 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. 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.

For anti-HIV evaluation of statins, compounds were tested for their anti-HIV activity and cytotoxicity in two independent laboratories (Fundació IrsiCaixa, Barcelona and Laboratory of Molecular Virology and Drug Discovery, Katholieke Universiteit Leuven, Belgium). Also, a similar assay was also repeated with MT-4 cells preincubated for 48 h with statins and the control compounds at 37ºC, and then washed twice with a phosphate-buffered saline (PBS) solution before infecting with HIV-1.

The virus titer in the MTT assay in MT-4 cells is calculated by an endpoint dilution technique. The maximum dilution in which the virus gives the minimum of absorbance is the one used to test the anti-HIV activity of screening compounds and in each evaluation there is a control titration of the virus used.

Anti-HIV assays in PBMC

PBMC from single donors in statin evaluations or PBMC pools in other evaluations were stimulated with 6 U/ml IL-2 (Roche) and 4 μg/ml phytohemagglutinin (PHA) during 72 h before use. After stimulation and during the performance of assays, only IL-2 at 10 U/ml was used. PBMC 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. Cell death was quantified by flow cytometry in forward (FSC) versus side scatter plots (SSC). Dead cells showed increased side and reduced forward scatter values compared with living cells. Anti-HIV activities in PBMC were performed at least three times. EC_{50} for each compound was calculated and considered valid when the variation between replicates was less than 4-fold.

Viral stocks were titrated and Tissue Culture Infectious Dose 50 ($TCID_{50}$) was calculated as described in [231], using the same PBMC pool used in the anti-HIV evaluating assays.

Anti-HIV assay in macrophages

The antiviral assay in monocyte-derived macrophages (MDM) was carried after 3 days of stimulation with M-CSF of monocytes. Cells were washed and incubated in complete culture medium containing different 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 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 assay in ex vivo lymphoid tissue culture

Anti-HIV activity in lymphoid tissue was evaluated as described before [232]. 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 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).

CHARACTERIZATION OF AN ANTI-HIV COMPOUND MODE OF ACTION

Evaluation of CD4+T cell death and HIV transfer

$2x10^5$ primary CD4+ T cells were cocultured with $2x10^5$ MOLT-4/CCR5 cells chronically infected with the X4 isolates NL4-3 and CI-1-SI or the R5 strain BaL. 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). CD4+ T cells and MOLT/CCR5 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. As a negative control, CD4+ T cells were also cocultured with MOLT/CCR5 uninfected cells.

Time of addition assays

MT-4 cells were infected with NL4-3 virus at a MOI 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 its EC_{50}). Virus production as quantity of p24 antigen in supernatant was determined 30 hours after infection [233].

Surface receptor staining and flow cytometry analysis

Staining of chemokine receptor CXCR4 and CCR5, and the CD45 and CD4 receptor was performed in CEM-CCR5 cell line. Briefly, $0.2x10^6$ 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 was acquired and percentage of positive cells and mean fluorescent intensity (MFI) were 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 CXCL12 was calculated for each condition.

Measurement of intracellular calcium concentrations

The intracellular calcium concentrations [Ca^{2+}] were determined as described previously [48]. 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). $1x10^6$ cells were first stimulated with dilution buffer (control) or test compound at various concentrations. As a second stimulus, CXCL12 (100 ng/ml), CCL3, CCL4 or CCL5 (1000 ng/ml) were used to induce [Ca^{2+}] signaling. The second stimulus was added 120 seconds after the first stimulus.

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 CXCL12 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, Perfect-Count Microspheres (Cytognos, Salamanca) were added and counted on a LSRII flow cytometer. Data was 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).

DEVELOPMENT AND CHARACTERIZATION OF RESISTANT HIV-1 STRAINS

Development of HIV-1 strains resistant to POL3026

$0.1x10^6$ MT-4 cells were incubated with the HIV-1 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 of two different cultured viruses when the concentration reached 0.034 µg/ml and 0.043 µg/ml for virus A (HP41resA) and virus B (HP38resB), respectively.

Growth kinetics of viruses

Parallel cultures of MT-4 cells exposed to the same MOI of virus (HXB2wt, HP41resA, HP38resB and the AMD3100-resistant viruses) were established. Infections were maintained during 5 days and supernatant was collected each day for p24 quantification with a commercial ELISA (Innotest^{TM} HIV-Ag; Innogenetics, Barcelona, Spain). 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 while untreated infected cultures (the wt HXB2.41, HP41resA and HP38resB) at a MOI 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 below.

Coreceptor switch assay

$1.5x10^5$ cells were infected with 13 ng of p24 antigen from BaL, CI1, CI2, CI3 and CI4 HIV-1. For propagation of the HIV-1 R5 CI5 (168.1 molecular clone) $5x10^6$ Sup-T1 were transfected with 2 μg proviral DNA using 0.4 cm cuvettes (BioRad), and 250 V and 950 μF.

Parallel cultures with different inhibitory conditions were maintained for each HIV-1 strain. Twice a week cell cultures were diluted 1/5 in fresh media containing or not the specific inhibitor. Concentrations for all drugs, except AMD3100 and POL3026 (1μg/ml), were adjusted to suboptimal concentrations to maintain a similar virus replication. 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^{TM} HIV-Ag; Innogenetics, Barcelona, Spain).

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

Viral phenotype determination

Coreceptor use was determined by evaluating the infectivity of the viruses in CCR5- or CXCR4- U87-CD4 cells. HIV strains to be tested are used to infect in duplicates and in parallel both types of U87-CD4 cells, which have been plated ($5x10^3$ cells/well, in a 96 well plate) the day before. One day post infection the cells are washed with PBS and day 7 post infection, p24 antigen in supernatant is quantified. The cells are fixed with PBS 1% formaldehyde and stained with Hoechst, and syncytia formation is checked. The X4 phenotype was confirmed also by evaluating virus infectivity in MT-2 cells as described in [231].

Sequencing (Env and RT)

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 PCR amplification of HIV *env*. Before sequencing the amplified DNA was purified with the QIAquick PCR Purification Kit (Qiagen, Barcelona, Spain).

The *env* gene (5514-8910) was amplified as previously described [233] with primers 5'-gataaagccacctttgcctagt-3' and 5'-ttctaggtctcgagatactg-3'. Nested PCR for the amplification of V1-V3 region (6586-7171) was performed using primer pairs 5'-aattaaccccactctgtgttagttta-3' and 5'-gctctccctggtcccctctgg-3'. The V3-V5 region (7045-7732) was amplified with primers 5'-ctgccaatttcacagacaatgc-3' and 5'-ctctttgccttggtgggtgcta-3' and sequenced with the ABI Prism™ BIGDYE Terminator 3.1 kit (Applied Biosystems, Madrid, Spain) in an ABI Prism 3100 *Avant* Genetic Analyzer. Sequences were analyzed with Sequencher v4.5 and edited with the BioEdit software. Amino acid positions were numbered according to the HXB2 strain (Los Alamos database).

CLINICAL EVALUATION

Simvastatin treatment of HIV+ patients

A pilot study was conducted in 12 HIV+ individuals attending HIV Clinical Unit of *Hospital Universitari Germans Trias i Pujol*. They were treated during 8 weeks with simvastatin, (Zocor®, 80 mg/day) in the absence of antiretroviral treatment. Virological and immunological status of patients was monitored at baseline and at weeks 4 and 8 after simvastatin introduction. A subset of patients was followed until week 12 of treatment. Eligible patients for the *in vivo* pilot study were those chronically HIV-infected subjects who had discontinued antiretroviral therapy at least 12 weeks before entry into the study and presented detectable plasma viral load (>1000 copies/ml). CD4+ T cell counts <250 cells/µl, viral load >100000 copies/ml, opportunistic infections, as well as creatinin values >1.5 than current values (II Degree) and liver enzyme levels more than 3 times above the upper normal limit (UNL), were considered exclusion criteria for the study.

All patients attending the HIV clinic between September 2004 and January 2005 that fulfilled the selection criteria were proposed to participate in this study and to start simvastatin 80 mg once daily. Written informed consent was obtained from all patients before enrollment. Quantitative analysis of HIV-1 RNA plasma levels were performed with the Amplicor HIV-1 Monitor, version 1.5 (Roche Molecular Systems, Branchburg, New Jersey, USA), at Covance Central Laboratory Services (Geneva, Switzerland). CD4+ and CD8+ T cell counts were assessed by standard flow cytometry and plasma cholesterol levels were determined.

Statistical analysis

The distribution of several variables measured during the simvastatin pilot study was analyzed using the Kolmorov-Smirnov test. The non-parametric Wilcoxon test for paired values was used to determine the statistical significant difference in these variables among different weeks (weeks 0, 4 and 8) of treatment with simvastatin. Significance was established at $P < 0.05$ (SPSS and Graph pad statistical software).

RESULTS

EVALUATION OF THE ANTI-HIV ACTIVITY OF STATINS *IN VITRO* AND EFFECT OF SIMVASTATIN TREATMENT IN HIV INFECTED PATIENTS

Abstract

Recent data suggest that statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, blocked HIV-1 replication *in vitro* and *in vivo*, which may have important implications for an alternative treatment of AIDS. We desired to delineate the potency, efficacy, specificity and cytotoxicity of statins *in vitro* and to assess their anti-HIV activity *in vivo*.

Anti-HIV activity of different statins (sodium and calcium salts of atorvastatin, lovastatin, simvastatin and fluvastatin and lactone forms of atorvastatin, lovastatin, simvastatin) was evaluated against the HIV-1 NL4-3 or BaL strains in the lymphoid MT-4 cell line or in PHA/IL-2 activated peripheral blood mononuclear cells (PBMC) purified from healthy donors. In parallel, a pilot study was conducted in 12 HIV+ individuals in the absence of antiretroviral treatment. Patients were treated during 8 weeks with simvastatin (80 mg/day), and their viral load, CD4+ T cell count and cholesterol levels were monitored.

We were unable to detect anti-HIV activity at subtoxic concentrations in MT-4 cells (CC_{50} range 0.8–5.6 μg/ml). Only modification of the standard anti-HIV assay allowed us to calculate a 50% effective concentration (EC_{50}) for lovastatin of 3.9 μg/ml albeit a 50 % cytotoxic concentration (CC_{50}) of 19 μg/ml. Similarly, due to the cytotoxicity, in PBMC we could only calculate an EC_{50} for simvastatin calcium salt (EC_{50} of 0.04 μg/ml and CC_{50} of 0.2 μg/ml). Treatment with simvastatin did not induce a significant change in viral load or in mean CD4 cell count in the study patients. Conversely, a significant decrease in plasma cholesterol levels was noted (mean change, – 47 mg/100 ml).

According to standard cell culture assays, the anti-HIV activity of the statins tested may be easily confounded by their accrue cytotoxicity at concentrations that would be required to block HIV replication. We did not observe any antiviral effect in HIV+ individuals after 8 weeks of treatment with simvastatin. Our results caution on the use of statins to treat HIV infection.

Statins and HIV

Statins could provide an interesting approach for the treatment of HIV-1 infection, therefore we decided to assess its mode of action, but first their anti-HIV activity in cell culture needed to be accurately measured. Preclinical drug screening is a necessary step that helps to delineate the potency, efficacy, cytotoxicity and specificity of a candidate compound and therefore allows to determine the necessary conditions for the study of the mechanism of action of active agents. The effect of lactone forms of atorvastatin, lovastatin, simvastatin and sodium and calcium salts of atorvastatin, lovastatin, simvastatin and fluvastatin was evaluated for their capacity to block the replication of HIV-1 by two independent laboratories. In this study we also evaluated the *in vivo* anti-HIV activity of simvastatin (Fig. 9) in a small group of HIV+ patients with detectable HIV RNA in plasma and that were off antiretroviral treatment for at least 3 months prior to initiation of our study.

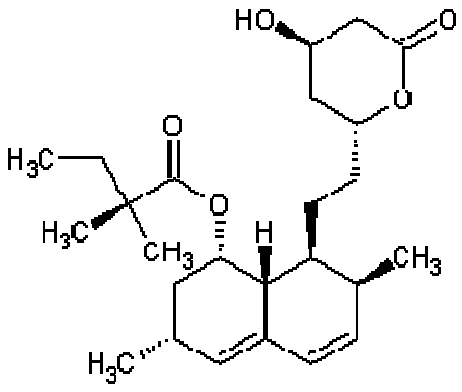


Figure 9. Simvastatine structure

Antiviral activity of statins against HIV-1 strains

To first test the antiviral activity and cytotoxicity of statins, we used a standard 5-day drug-screening assay in the lymphoid MT-4 cell line that is generally used in our laboratory for the throughput evaluation of candidate antiviral agents, in which parallel cytotoxic concentrations in the absence of virus infection are tested [230,234].

We found that the different statins, either in their lactone forms or as their sodium or calcium salts, did not show anti-HIV activity at subtoxic concentrations. Conversely, compounds known for their anti-HIV activity, which were used as controls (the reverse transcriptase inhibitor AZT, the virus binding inhibitor dextran sulfate [235], or the CXCR4 antagonist AMD3100 [140], were clearly active (EC_{50}: 0.002, 0.07 and 0.003 μg/ml, respectively) at concentrations well below their 50% cytotoxic concentration (CC_{50}: > 1, > 125 and > 5, respectively). The CC_{50} values in MT-4 cells of the statins tested ranged from 0.8 to 5.6 μg/ml. The EC_{50} and CC_{50} values of all tested compounds are shown in Table 2.

In order to reduce the level of toxicity described above, we shortened cell exposure to statins and control compounds by preincubating the cells during 48 h at 37 ºC and washing them before infecting them with HIV-1 NL4-3. We were able to detect the anti-HIV activity of lovastatin (EC_{50} 3.9 μg/ml) despite detectable cytotoxicity (CC_{50}: 19 μg/ml). All other statins tested did not show anti-HIV activity at subtoxic concentrations.

A similar result was found when statins were evaluated in peripheral blood mononuclear cells. That is, with the exception of simvastatin calcium salt, an EC_{50} value could not be calculated below cytotoxic concentrations (CC_{50} range 0.2-15.2 μg/ml). The EC_{50} for simvastatin was calculated at 0.04 μg/ml. However, we could not reach 100% protection and accrued cytotoxicity was found at 0.2 μg/ml. We concluded that the anti-HIV activity of statins may easily be confounded by their cytotoxicity at concentrations that would be required to block HIV replication.

Anti-HIV evaluations of these statins were also performed by an independent laboratory (Myriam Witvrouw, PhD) and similar results were obtained.

Table 2. Anti-HIV NL4-3 and BaL activity and cytotoxicity of different statins and their sodium and calcium salts.

Compound	EC_{50} (µg/ml) [a]		CC_{50} (µg/ml) [b]	
	HIV-1 NL4-3 MT-4 cells	HIV-1 BaL PBMC	MT-4 cells	PBMC
Atorvastatin (lactone)	>1,03	>9,6	1,03	9,6
Lovastatin	>1,8	>6,1	1,8	6,1
Mevinolin (Sigma lovastatin)	>0,9	>10,5	0,9	10,5
Simvastatin	>6,1	>9,6	6,1	9,6
Atorvastatin Na	>5,6	>5,5	5,6	5,5
Lovastatin Na	>1,2	>15,2	1,2	15,2
Simvastatin Na	>0,87	>11,9	0,87	11,9
Fluvastatin Na	>0,82	>8,1	0,82	8,1
Atorvastatin Ca	>2,7	>9,6	2,7	9,6
Lovastatin Ca	>0,85	>12,8	0,85	12,8
Simvastatin Ca	>0,80	0,04	0,80	0,2
Fluvastatin Ca	>0,98	>11,9	0,98	11,9
AMD 3100	0,003	NT	>5	NT
AZT	0,002	<0,008	>1	>1
Dextran sulfate	0,07	14,6	>25	>125

[a] EC_{50} : 50% effective concentration, or concentration needed to inhibit 50% HIV-induced cell death, evaluated with the MTT method in MT-4 cells or through the production of HIV p24 antigen by acutely infected PBMC.

[b] CC_{50} : 50% cytotoxic concentration or concentration required to induce 50% death of non-infected cells, evaluated with the MTT method in MT-4 cells or through cell morphology changes in PBMC after flow cytometry analysis.

NT: not tested

Antiretroviral activity of simvastatin in a pilot study

To evaluate the antiviral properties of statins *in vivo*, a pilot study was conducted in 12 HIV+ individuals in the absence of other antiretroviral treatment. Participating patients had interrupted antiretroviral treatment for at least 12 weeks prior to initiation of simvastatin treatment (80 mg once daily) for up to 8 weeks. This pilot study was conducted in the HIV Clinical Unit of *Hospital Germans Trias i Pujol*.

It had been suggested that the anti-HIV effect of statins was not due to the inhibition of cholesterol biosynthesis and the consequent reduction of cholesterol levels in plasma [2]. However, cholesterol levels were taken as a control for the efficacy of treatment and thus, were used to correlate the effect of statin treatment with anti-HIV efficacy. Basal mean viral load (VL) was 29108 ± 34185 RNA copies/ml (4.14 ± 0.72 log RNA copies/ml) and mean CD4 cell count 491±187 cells/µl. Mean values of virological and immunological conditions, total HDL- and LDL-cholesterol levels at weeks 0 and 4 and 8 are shown in Table 3. Viral load, CD4 T cell count and cholesterol plasma level of each patient at weeks 0, 4, 8 and 12 are shown in Table 4.

There were no significant (P: 0.5771) changes in mean VL after 4 weeks of treatment with simvastatin. However, 2 patients reached a reduction of 0.47 log RNA copies/ml and a weak decrease in VL was noted in 6 patients that, however, did not reach statistical significance. A rise in VL was noted in 2 patients (0.82 and 0.50 log RNA copies/ml, respectively). Mean CD4+ T cell count increased slightly but did not reach statistical significance and a drop in the CD4 T cell counts observed in 5 out of 10 patients. A change in mean plasma total cholesterol level was noted. There was a significant decrease (P: 0.0049) between baseline and week 4 (172 ± 45 and 128 ± 17 mg/100 ml, respectively). More precisely, plasma cholesterol was reduced in 9 out of 11 subjects after 4 weeks of treatment when compared to their cholesterol levels at initiation of simvastatin treatment. Similarly, mean LDL-cholesterol levels decreased significantly (P: 0.0049) at week 4, particularly in 10 out of 11 patients. Conversely, there was no significant change in the HDL-cholesterol levels (P: 0.1602).

Similar results were observed at week 8. There was no significant change in mean viral load in comparison to the basal values (P: 0.4316) and only 1 patient out of 11 had a relevant decrease (–0.55 log copies/ml), while 3 patients had an increase in VL (0.89, 1.68 and 0.52 log copies/ml, respectively). There was no significant (P: 0.250) change in mean CD4 cell count as compared to basal levels. On the contrary, mean

plasma total cholesterol levels and LDL-cholesterol levels were significantly reduced (P: 0.0078 and P: 0.0078, respectively) in the absence of a change in mean HDL-cholesterol (P: 0.7422) confirming the efficacy of simvastatin as a cholesterol-reducing agent in HIV+ individuals.

A subset of patients (n: 8) continued simvastatin treatment for up to 12 weeks (VL, CD4 T cell counts and cholesterol plasma levels are shown in Table 4). No significant reduction in plasma VL or CD4+ T cell count was noted despite a significant reduction in cholesterol levels.

Table 3. Virological and immunological variables of HIV+ individuals treated with simvastatin (80 mg/day)

Variable	week 0	week 4	week 8	Wilcoxon test P value	
				Weeks 4-0	Weeks 8-0
Viral Load					
No. of subjects with data	12	12	11		
Mean copies/ml ± SD	29108 ± 34185	24013 ± 22695	35837 ± 49400		
(log copies/ml)	(4.14)	(4.10)	(4.27)	0,577	0,432
CD4 T cell count					
No. of subjects with data	10	11	9		
Mean cells/ml ± SD	491 ± 187	520 ± 167	505 ± 182	0,734	0,25
Cholesterol					
No. of subjects with data	11	12	9		
Mean mg/100 ml ± SD	172 ± 45	128 ± 17	125 ± 8	0,005	0,008
HDL					
No. of subjects with data	11	12	9		
Mean ± SD	38 ± 9	35 ± 6	35 ± 4	0,16	0,742
LDL					
No. of subjects with data	11	12	9		
Mean ± SD	110 ± 41	70 ± 18	73 ± 12	0,005	0,008

Table 4. Viral load, CD4 T cell counts and cholesterol plasma level of the 12 patients HIV+ during the simvastatin treatment.

Patient	Variable	week 0	week 4	week 8	week 12
1	Viral load (copies/ml)	24500	76900	67300	92188
	CD4 T cell count (cells/ml)	544	424	430	460
	Cholesterol (mg/dl)	NA[a]	NA	NA	NA
2	Viral load (copies/ml)	12800	11400	12800	NA
	CD4 T cell count (cells/ml)	617	530	548	592
	Cholesterol (mg/dl)	155	139	128	116
3	Viral load (copies/ml)	4910	7070	4250	NA
	CD4 T cell count (cells/ml)	NA	683	657	NA
	Cholesterol (mg/dl)	93	93		NA
4	Viral load (copies/ml)	18800	8480	10400	13600
	CD4 T cell count (cells/ml)	866	662	859	NA
	Cholesterol (mg/dl)	135	112	112	NA
5	Viral load (copies/ml)	200	200	9580	52600
	CD4 T cell count (cells/ml)	460	424	376	303
	Cholesterol (mg/dl)	283	143	135	147
6	Viral load (copies/ml)	32600	15700	15700	NA
	CD4 T cell count (cells/ml)	334	388	NA	NA
	Cholesterol (mg/dl)	101	112	NA	NA
7	Viral load (copies/ml)	5050	33600	39300	39300
	CD4 T cell count (cells/ml)	NA	835	NA	NA
	Cholesterol (mg/dl)	182	128	NA	NA
8	Viral load (copies/ml)	51400	32600	171000	55100
	CD4 T cell count (cells/ml)	207	250	261	NA
	Cholesterol (mg/dl)	170	120	124	NA
9	Viral load (copies/ml)	23100	7810	6560	5140
	CD4 T cell count (cells/ml)	560	503	545	NA
	Cholesterol (mg/dl)	NA	128	120	NA
10	Viral load (copies/ml)	6840	6390	NA	7420
	CD4 T cell count (cells/ml)	302	402	336	268
	Cholesterol (mg/dl)	170	124	116	128
11	Viral load (copies/ml)	44100	45900	49900	17000
	CD4 T cell count (cells/ml)	452	621	NA	462
	Cholesterol (mg/dl)	155	170	NA	124
12	Viral load (copies/ml)	125000	42100	NA	46100
	CD4 T cell count (cells/ml)	572	NA	569	NA
	Cholesterol (mg/dl)	182	116	128	NA

[a] NA: Not available

HIV-1 ESCAPE OF CCR5 CORECEPTOR ANTAGONISM THROUGH SELECTION OF CXCR4-USING VARIANTS *IN VITRO*

Abstract

HIV-1 coreceptor switch from CCR5 to CXCR4 is associated with disease progression and AIDS. Selection of resistant HIV-1 to CCR5 agents in cell culture has often occurred in the absence of coreceptor switch. With CCR5 antagonists currently in clinical trials, their impact on coreceptor use is still in doubt.

Six R5 HIV-1 strains were passaged in lymphoid cells expressing high CXCR4 and low CCR5, in the absence or presence of CCR5 inhibitors (TAK-779, mAb 2D7 and CCL5). AMD3100, AZT and 3TC were used as controls. Phenotype and genotype changes as well as virus coreceptor use were evaluated.

In the absence of drug pressure, three out of six strains expanded their coreceptor use to CXCR4 at different times, suggesting that not all virus strains had the capacity to do so. Lowering the replication rate with a suboptimal concentration of different anti-HIV agents (RT inhibitors or CCR5 agents) delayed coreceptor switch. However, virus breakthrough was observed earlier in the presence of CCR5-targeting agents than in presence of RT inhibitors and was associated to a change in sensitivity to TAK-779 or AMD3100, virus coreceptor expansion to CXCR4 and changes in the V3 loop region of gp120.

Our results suggest that HIV-1 may escape CCR5 drug pressure through coreceptor switch. Experimental conditions strongly determine the outcome of CCR5 drug pressure in cell culture. A cell culture model of the evolution of HIV-1 coreceptor use may be relevant to assess the propensity of clinical isolates to develop resistance through coreceptor change.

Characterization of viral strains for selection with CCR5 inhibitors

Six HIV-1 strains (CI1-CI5 and the laboratory–adapted BaL strain) were selected by their R5 phenotype. Tropism was determined by assessing their growth in MT-2 cells and in U87-CD4 cells expressing the appropriate coreceptor (Table 5). The selected HIV-1 strains were titrated in PBMC and their susceptibility to CXCR4, CCR5 and reverse transcriptase inhibitors (AMD3100, TAK-779 and AZT, respectively) was evaluated (Table 5). As expected, none was sensitive to AMD3100 at the maximum concentration tested (1µg/ml). All strains were sensitive to TAK-779 and all but one (CI4) had similar sensitivity to AZT.

Table 5. Inhibition of virus replication and coreceptor use of different HIV-1 strains

Virus	EC_{50} [a] (µg/ml)			Coreceptor use [b]		
	AZT	AMD3100	TAK-779	MT-2	U87 CCR5+	U87 CXCR4+
BaL	0,001	>1	0,004	-	+	-
CI1	0,0001	>1	0,002	-	+	-
CI2	0,0002	>1	0,0001	-	+	-
CI3	0,0001	>1	0,0001	-	+	-
CI4	>0,5	>1	0,0001	-	+	-
CI5	0,0006	>1	0,0001	-	+	-

[a] EC_{50} : 50% effective concentration, or concentration needed to inhibit 50% HIV p24 antigen production, evaluated in PHA and IL2 stimulated PBMC. Maximum concentration tested was 0.5 µg/ml for AZT and 1 µg/ml for AMD3100 and TAK-779.

[b] Coreceptor use was determined by evaluation of syncytium formation in the corresponding cell line and determination of p24 antigen in the cell supernatant (data not shown).

Emergence of HIV-1 coreceptor switch variants *in vitro*

Virus were passaged in SupT-1 cells which express high levels of CXCR4 and low levels (undetectable by flow cytometry but positive by western blot analysis, data not shown) of CCR5 coreceptor [236]. During successive passages viral replication was

reminiscent of slow replicating, non-syncytium inducing phenotype. In the absence of drug pressure, three out of six strains (CI3, CI4 and CI5) were able to switch from the R5 to R5/X4 phenotype (Fig. 10, Table 6). Change of phenotype correlated with the observation of syncytia in cell cultures and increased replication rate, as measured by p24 antigen in the supernatant (Fig. 10). After the peak of p24 production there was a drop in p24, probably caused by the massive cell death observed after the spread of R5X4/X4 variants (Fig. 10).

The emergence of R5X4 variants from the CI5 culture took place after 4 passages (14 days) whereas syncytia formation in CI3 and CI4 cultures could be detected at passage 17 (59 days) and 30 (105 days), respectively. Identical experiments were repeated four times with CI5 and twice with CI3 to ensure the reproducibility of coreceptor switch (data not shown). In all cases, the coreceptor switch variants from CI5 and CI3 appeared within 10 to 16 days and 63 to 77 days, respectively. The two other clinical isolates (CI1, CI2) and BaL, did not switch coreceptor, despite being 300 days in culture.

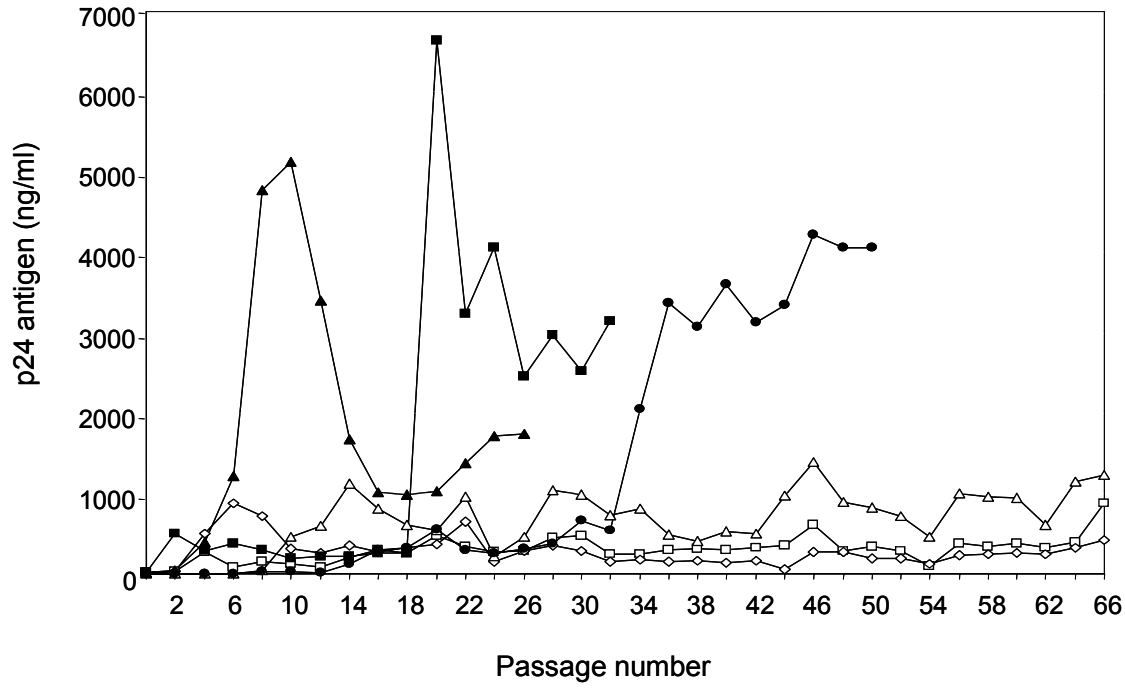


Figure 10. Growth of R5 HIV-1 strains in Sup-T1 cells. Cells were infected with the corresponding strain and replication was measured as p24 antigen in the supernatant of cells. Virus replication was reminiscent of R5 slow replication phenotype. Three out of six strains, CI3 (■), CI4 (●) and CI5 (▲), showed increased replication at different times after initiation of infection, which correlated with the gain of CXCR4 use. Conversely, BaL (□), CI1 (◊) and CI2 (Δ) maintained a slow replication phenotype.

CCR5 antagonists accelerated the emergence of CXCR4-using variants compared to RT inhibitors

As explained above, passages of the 6 HIV-1 strains occurred at relatively slow replication rates due to the low availability of CCR5 coreceptor. At the same time, similar cultures were passaged in presence of the RT inhibitors, AZT or 3TC, and the CCR5 antagonist TAK-779 at suboptimal concentrations, applying similar pressure on the virus but on different target genes. The gain of CXCR4 use by the three isolates described above was delayed with AZT, 3TC and TAK-779 (Fig. 11). However, in both CI3 and CI5, CXCR4-using viruses emerged earlier with TAK-779 compared to the cultures with AZT or 3TC. (Fig. 11A, 11C and Table 6). For CI3, emergence of CXCR4-using variants in the presence of TAK-779 ($CI3_{TAK-779}$) was delayed for 15 passages (49 days) compared to the untreated control ($CI3_C$), whereas AZT ($CI3_{AZT}$) delayed it for 21 passages (70 days). The CI5 strain cultured with TAK-779 ($CI5_{TAK-779}$) switched coreceptor 5 to 9 passages (17 to 59 days) after the CI5 without drug ($CI5_C$), depending on the experiment. Coreceptor switch variants of CI5 in presence of AZT ($CI5_{AZT}$) could not be detected even 18 or 33 passages (63 or 115 days) after their detection in the control cultures.

CI4 in presence of AZT ($CI4_{AZT}$) switched coreceptor earlier than in presence of TAK-779 ($CI4_{TAK-779}$) (Fig. 11B and Table 6). This virus strain was shown resistant to AZT by phenotype (Table 5) and genotype testing (data not shown). The emergence of X4 viruses in the presence of AZT ($CI4_{AZT}$) took place only 3 passages (11 days) after detection of CXCR4-using variants in the cultures without drugs ($CI4_C$).

A parallel culture of each strain was maintained with AMD3100 (1µg/ml). AMD3100 prevented the emergence of CXCR4-using viruses in the cultures of the three clinical isolates that switched in the absence of drug pressure.

Selection of the R5X4 phenotype could also be induced with the mAb anti-CCR5 2D7 and CCL5 (Fig. 12). The switch of coreceptor use was delayed if compared to the untreated culture, but was noticed earlier with all CCR5 agents when compared to cultures growing at a similar replication rate (in the presence of AZT or 3TC).

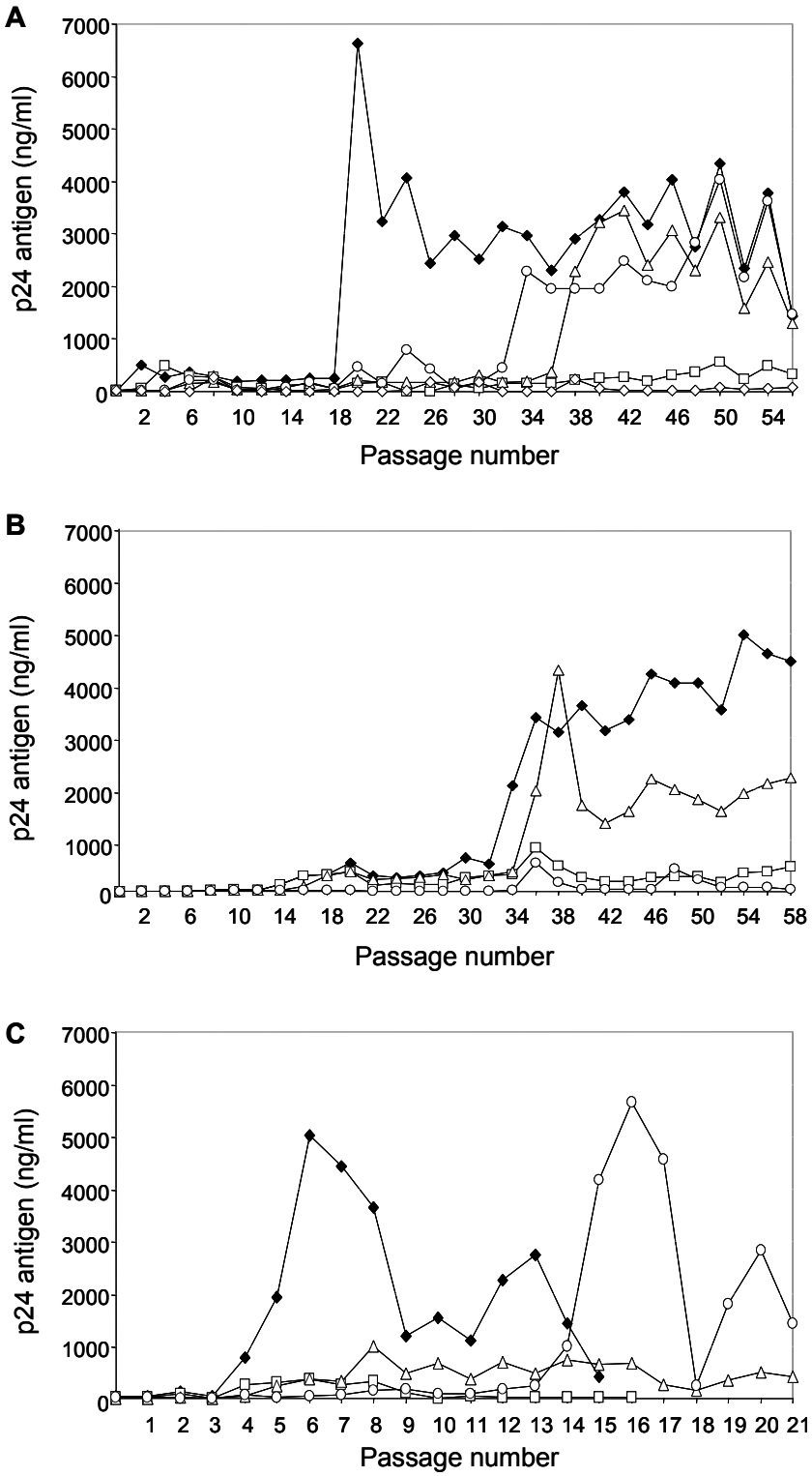


Figure 11. Growth of HIV-1 strains in the presence of HIV-1 inhibitors. Cells were infected with the corresponding strain and virus replication was measured as p24 antigen in the supernatant of cells. The emergence of R5X4 variants is linked to a high increase in the p24 antigen. Each parental HIV-1 isolate CI3 **(A)**, CI4 **(B)**, CI5 **(C)** was passaged without drug (♦), with AMD3100 (□), AZT (Δ) orTAK-779 (○). CI3 (A) was also passaged with 3TC (◊). For CI3 (A) and CI5 (C), peak of replication was first noticed under TAK-779 than AZT or 3TC selective pressure and similar results were obtained for 3 independent experiments.

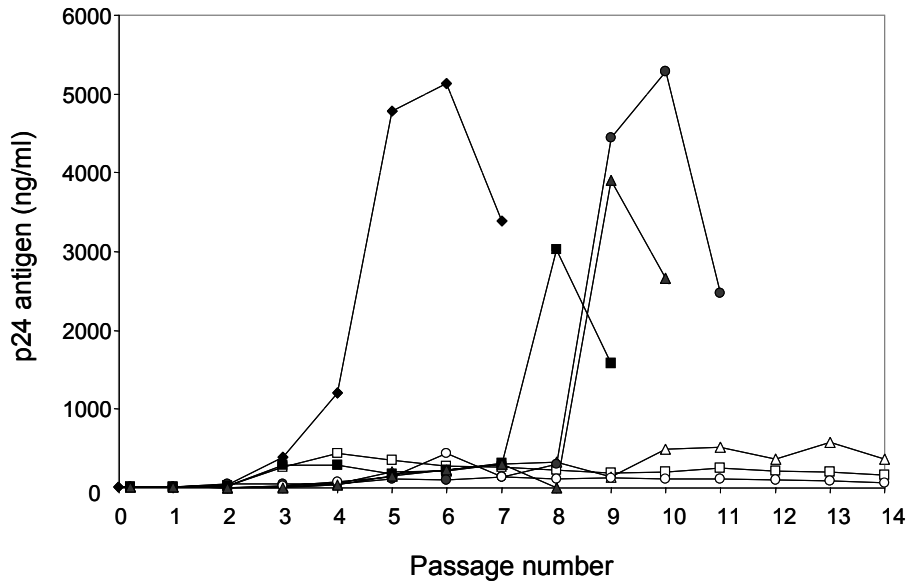


Figure 12. Growth of HIV-1 CI5 in the presence of different CCR5-targeting compounds. Filled symbols represent the variants that gained CXCR4 use, open symbols represent the variants that did not change tropism. Without drug (♦) or with TAK-779 (●), mAb 2D7 (■), CCL5 (▲), AMD3100 (□), AZT (Δ) and 3TC (○).

Tropism change accompanied by reduced sensitivity to TAK-779

The sensitivity to AZT, AMD3100 and TAK-779 of each parental virus and all the viruses obtained after the passages was determined in PBMC. EC_{50} values are shown in Table 6. As expected, almost all the viruses that gained CXCR4 usage, were less sensitive to TAK-779. The control CI5 virus ($CI5_C$), of R5X4 phenotype, was 30-fold less sensitive to TAK-779 compared to the parental CI5 (EC_{50} 0.003 µg/ml and 0.0001 µg/ml, respectively). Similarly, the EC_{50} of TAK-779 for $CI5_{TAK\text{-}779}$ and $CI5_{CCL5}$ increased 30-fold and 100-fold for the $CI5_{2D7}$ strain. Comparable results were obtained with the CI3 virus of which switched variants were 40-fold ($CI3_C$), 90-fold ($CI3_{AZT}$) and 60-fold ($CI3_{TAK\text{-}779}$) less sensitive to TAK-779. Nevertheless, the R5X4 variant $CI4_{AZT}$ was as sensitive as the CI4 parental isolate. Concerning the AMD3100 inhibition, parental isolates were totally resistant, but the R5X4 variants gained some sensitivity. However, an EC_{50} value for AMD3100 could not be calculated at the concentrations used, except in two cases, for $CI3_C$ (0.03 µg/ml) and for $CI4_C$ (0.1 µg/ml). Drug sensitivity in primary cells is prone to higher variation in experimental error and variation in virus titre may explain the 10-fold increase in the AMD3100, AZT and 3TC CI5 passaged strains.

Table 6. Inhibition of virus replication and coreceptor use of HIV-1 stains cultured in the presence of different HIV entry inhibitors

Virus	Culture conditions	Passage Nº [a]	EC_{50} [b] (µg/ml)			Coreceptor use [c]		
			AZT	AMD3100	TAK-779	MT-2	U87 CCR5+	U87 CXCR4+
$CI3_{parental}$	-	-	0,0001	>1	0,0001	-	+	-
$CI3_{C}$	no drug	17	0,001	0,03	0,004	+	+	+
$CI3_{AMD3100}$	AMD3100	-	0,0001	>1	0,0001	-	+	-
$CI3_{AZT}$	AZT	38	0,001	>1	0,009	+	+	+
$CI3_{TAK-779}$	TAK-779	32	0,0007	>1	0,006	+	+	+
$CI3_{3TC}$	3TC	-	0,001	>1	0,0004	-	+	-
$CI4_{parental}$	-	-	>0,5	>1	0.0001	-	+	-
$CI4_{C}$	no drug	30	0,008	0,1	0,002	+	+	+
$CI4_{AMD3100}$	AMD3100	-	0,09	>1	0,0004	-	+	-
$CI4_{AZT}$	AZT	33	>0,5	>1	0,0001	+	+	+
$CI4_{TAK-779}$	TAK-779	-	>0,5	>1	0,0001	-	+	-
$CI5_{parental}$	-	-	0,0006	>1	0,0001	-	+	-
$CI5_{C}$	no drug	3	0,002	>1	0,003	+	+	+
$CI5_{AMD3100}$	AMD3100	-	0,001	>1	0,001	-	+	-
$CI5_{AZT}$	AZT	-	0,001	>1	0,001	-	+	-
$CI5_{TAK-779}$	TAK-779	8	0,002	>1	0,003	+	+	+
$CI5_{CCL5}$	CCL5	8	0,001	>1	0,003	+	+	+
$CI5_{2D7}$	2D7	7	0,0005	>1	0,01	+	+	+
$CI5_{3TC}$	3TC	-	0,002	>1	0,001	-	+	-

[a] Passage number at which the outgrow of CXCR4-using variants was first detected. Values represent the mean of 2 separate evaluations.

[b] EC_{50}: 50% effective concentration, or concentration needed to inhibit 50% HIV p24 antigen production, evaluated in stimulated PBMC. Maximum concentration tested was 0.5 µg/ml for AZT and 1 µg/ml for AMD3100 and TAK-779. Values represent the mean of 2 separate evaluations done in triplicate.

[c] Coreceptor use was determined by evaluation of syncytia formation in the corresponding cell line and determination of p24 antigen in the cell supernatant (data not shown).

Genotypic changes in gp120 of coreceptor switch variants

Amino acid changes associated with virus coreceptor switch are shown in Fig. 13. Most of the mutations occurred in the V3 loop and substitutions that generated the positively charged amino acids Arg (R) or Lys (K) were heavily favoured, mainly in V3 (e.g. $CI4_C$, $CI5_C$, $CI5_{TAK\text{-}779}$), but also in V2 ($CI4_C$). Changes in the V3 loop net charge have been previously associated with coreceptor switch [80,81,237]. We observe a potential glycosylation site loss in V3 at position 301 ($CI5_C$, $CI5_{TAK\text{-}779.2}$, $CI5_{2D7}$), that has also been associated with coreceptor switch [89]. No other mutations in the gp120 coding region were observed (data not shown). Notably, almost all viral strains that switched coreceptor had a glycine (G) to arginine (R) mutation at position 314 in V3 loop. However, two patterns of mutations were selected from CI5 passaged with TAK-779 ($CI5_{TAK\text{-}779.1}$ and $CI5_{TAK\text{-}779.2}$). In one experiment $CI5_{TAK\text{-}779.1}$ showed the emergence of an aspartic acid (D) to an asparagine (N) at position 322 in V3, increasing the V3 net positive charge and the loss of the N-linked glycosylation site at position 301.

Virus	Amino Acid position																												
	V1		V2			C2								V3								C3			V4	C4	V5		
	130	143	167	171	174	220	275	276	277	283	288	292	301	303	306	313	314	316	321	322	351	359	365	410	429	462	470		
CI3 PARENTAL	N	T	D	R	A	P	E	N	F	T	L	V	N	T	S	P	G	A	G	Q	E	I	P	-	E	N	P		
CI3 C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	S	-	-	-	-		
CI3 AMD3100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
CI3 AZT	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-		
CI3 TAK-779	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S/G	-	R	P/T	-	-	-	-	S	-	-	-	-		
CI3 3TC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
CI4 PARENTAL	N	T	G	T	S	G	E	N	F	T	L	V	N	T	G	P	G	T	N	I	K	I	S	T	K	N	P		
CI4 C	-	-	G/R	-	S/R	-	-	-	-	-	L/V	-	-	K	-	-	R	-	-	-	-	L	-	-	E	-	-		
CI4 AMD3100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
CI4 AZT	-	-	R	T/A	-	-	-	-	-	-	-	-	-	-	-	A	R	-	-	-	R	-	-	-	E	-	-		
CI4 TAK-779	-	-	-	-	-	-	A	-	-	A	-	I	-	-	-	-	-	-	D	-	R	-	-	-	E	-	-		
CI5 PARENTAL	C	S	G	K	A	P	V	V	F	T	L	V	N	T	S	P	G	A	G	D	E	I	S	G	K	N	P		
CI5 C	-	S/N	-	-	-	C/T	-	-	-	-	-	-	K	-	-	-	R	-	-	-	-	-	-	-	-	-	-		
CI5 AMD3100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
CI5 AZT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
CI5 3TC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
CI5 2D7	Y/C	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	R	-	-	-	-	-	-	R/G	-	Y/N	-		
CI5 CCL5	Y/C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-		
CI5 TAK-779.1	-	-	-	-	-	-	-	V	-	-	-	-	-	T/I	-	-	-	-	-	N	-	-	-	-	-	-	-		
CI5 TAK-779.2	Y/C	-	-	-	-	-	-	-	-	-	-	-	K/N	T/K	-	-	R	-	-	-	-	-	-	-	-	-	P/L		

Figure 13. Comparative analysis of the consensus gp120 amino acid sequence from the parental and passage-derived HIV-1 strains. Amino acid positions are numbered according to HXB2 start of gp120. (-) represent homology with the parental strain.

ANTI-HIV ACTIVITY AND RESISTANCE PROFILE OF THE CXCR4 ANTAGONIST POL3026

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 CXCL12-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.

The novel CXCR4 antagonist POL3026

Recently, highly potent and selective β-hairpin mimetic CXCR4 inhibitors with good pharmacokinetic profiles have been described [226]. 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 (Fig. 14). In this study we wanted to determine the anti-HIV activity, specificity and mode of action of POL3026.

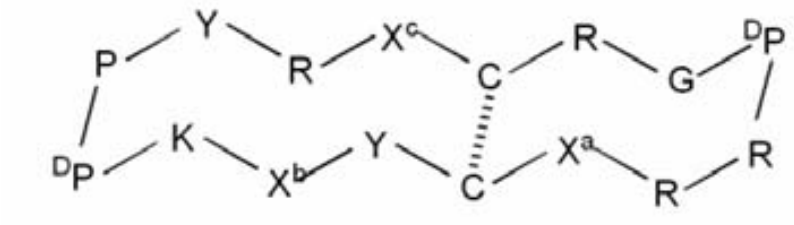


Figure 14. Structure of POL3026. X^a =2-Nal; X^b =Q; X^c= Cit

Potent anti-HIV activity of POL3026 against a broad panel of HIV strains

We used the standard drug-screening assay with MT4 cells based on the MTT method that is generally used in our laboratory for the throughput evaluation of candidate antiviral agents [230,234]. As shown in Table 7, 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) (Tables 7 and 8). 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 7 and 8. A summary of mean EC_{50} values and SD values for all evaluations done during this study are shown in the Supplementary Table 1 and 2 , respectively.

Table 7. Anti-HIV activity of POL3026 against wt NL4-3 and different resistant strains

Compound	EC_{50} (µg/ml) [a]								CC_{50} (µg/ml) [b]
	NL4-3 wt	K103N	IRLL98DRT	Y181C	Y188L	CI-1-SI	T-20r/C34r	HIV-2 ROD	no virus
AZT	0,0008	0,0005	0,0006	0,0002	0,0007	0,4	0,0005	0,0005	>1
AMD3100	0,001	0,001	0,001	0,002	0,001	0,004	0,001	0,001	>5
Nevirapine	0,004	0,03	>2	0,9	>2	0,004	-	>2	>2
Efavirenz	0,0004	0,001	>0,1	0,002	>0,1	0,0001	-	>0,1	>0,1
POL3026	0,0001	0,0001	0,00001	0,0001	0,00005	0,0002	0,0004	0,0001	>1
DS	0,009	-	-	-	-	5,4	0,08	-	>125
C34	0,005	-	-	-	-	-	0,2	-	>5
T-20	0,1	-	-	-	-	-	1	-	>5
CXCL12	0,07	-	-	-	-	-	0,1	-	>5

[a] EC_{50}: Effective concentration 50 or the concentration needed to inhibit by a 50% the MT4 cell death as measured by the MTT assay. Results shown are the mean of three separate experiments performed in triplicates. SD for all values is shown in supplementary table 2.

[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.

Table 8. Anti-HIV activity of POL3026 against HIV-1 strains from different subtypes

Compound	EC_{50} (µg/ml) [a]								CC_{50} (µg/ml) [b]
HIV Strain Sub-type	NL4-3	92BR014 B	UG92029 A	39RW024 A	92UG021 D	92UG024 D	93BR020 F	BCF06 O	No virus
AZT	0,001	0,001	0,001	0,001	0,001	0,0009	0,002	0,0005	>0,1
Nevirapine	0,004	0,01	0,05	0,04	0,08	0,1	0,09	0,09	>1
C34	0,003	0,00003	0,0001	0,0006	0,005	0,002	0,01	0,0002	>1
T-20	0,3	0,007	0,007	0,0003	0,002	0,003	0,004	0,00004	>2
AMD3100	0,001	0,004	0,008	0,001	0,002	0,008	0,004	0,004	>1
CXCL12	0,07	>1	>1	>1	>1	>1	>1	>1	>1
POL3026	0,00006	0,00001	0,0001	0,00002	0,0001	0,0002	0,00003	0,00008	>0,1

[a] EC_{50}: Effective concentration 50 or the concentration needed to inhibit by a 50% the MT4 cell death as measured by the MTT assay. Results shown are the mean of three separate experiments performed in triplicates. SD for all values is shown in supplementary table 2.

[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.

POL3026 was active against R5X4 and X4 strains in PBMC

Dualtropic (R5X4) HIV-1 strains preferentially use CXCR4 as entry coreceptor [238]. 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 9, Supplementary Table 1 and 2 for SD) with the exception of HIV-1 CI6 that was 6-fold less sensitive to POL3026 as compared to the NL4-3 strain 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.

Table 9. Anti-HIV activity of POL3026 against X4 and R5X4 strains in PBMC

Compound	EC_{50} (µg/ml)[a]						CC_{50} (µg/ml)[b]
	NL4-3 wt	BaL	89.6	CI6	CI7	CI8	no virus
AZT	0,001	0,0003	0,0006	0,001	0,001	0,00003	>0,1
TAK-779	>0,2	0,006	>0,2	>0,2	>0,2	>0,2	>0,2
AMD3100	0,006	>0,5	0,008	0,05	0,04	0,05	>0,5
T-20	0,2	0,003	0,3	0,2	0,2	0,2	>0,5
POL3026	0,005	>0,1	0,007	0,03	0,002	0,0005	>0,1

[a] EC_{50}: 50% effective concentration, or concentration needed to inhibit by a 50% the replication of HIV strains as measured by HIV p24 antigen production, evaluated in PHA and IL2 stimulated PBMC. Results shown are the mean of three separate experiments performed in triplicates. SD for all values is shown in supplementary table 2.

[b] CC_{50}: 50% cytotoxic concentration or concentration required to induce 50% death of non-infected cells, evaluated by flow cytometry in forward versus side scatter plots.

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 [225]. In this assay we measured the single-cell-death of target cells (CD4+ T cells) induced by effector cells that expressed different Env (chronically

infected MOLTCCR5+ cells) and also the p24 transfer to target cells. POL3026 efficiently blocked single-cell-death of CD4+ T cells induced by MOLT-4/NL4-3 and MOLT-4/CI-1-SI cells expressing X4 HIV-1 glycoproteins (Fig. 15) 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. 15). As previously shown [225], 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.

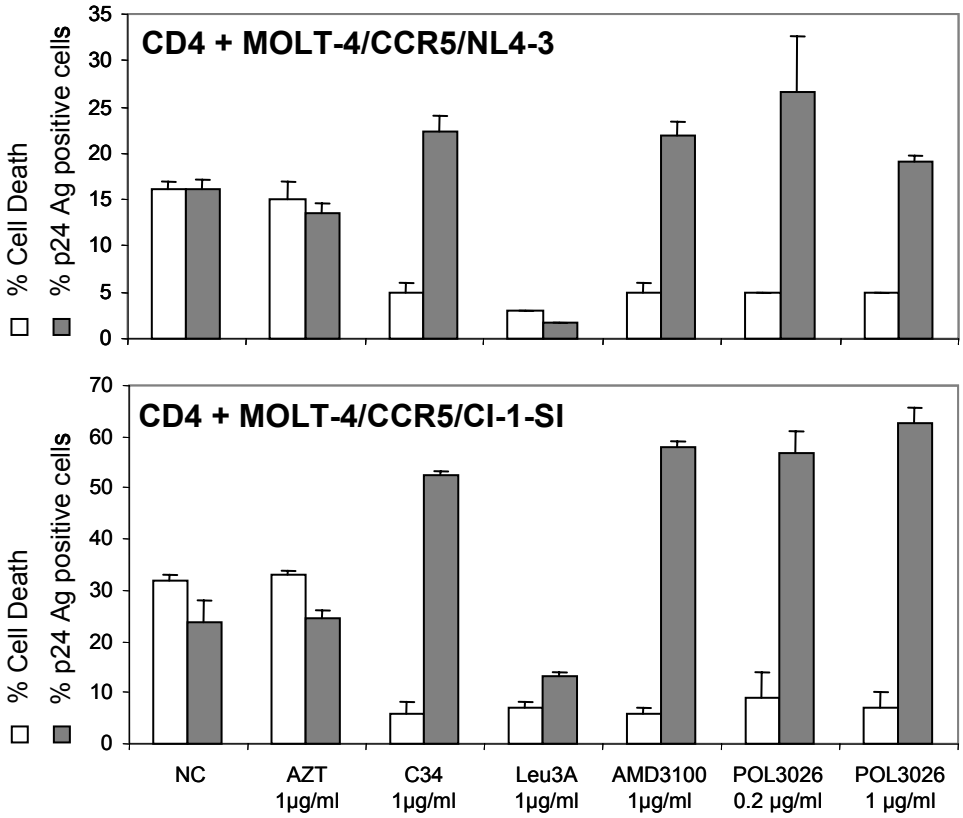


Figure 15. Single-cell-death and p24 transfer. Effect of compounds (RT inhibitor AZT, fusion inhibitor C34, anti-CD4 mAb Leu3A, and CXCR4 antagonists AMD3100 and POL3026) on single cell death and p24 transfer to CD4+ T cells, induced by coculture with two different sets of X4 HIV-1 (NL4-3 and CI-1-SI) 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.

POL3026 acted as a CXCR4 antagonist in a time of addition assay

In time of drug addition experiments, an infection synchronized by temperature is established and compounds are added at different times post-infection. Virus production is measured after one cycle of replication. As shown in Fig. 16, similar to the CXCR4 agents AMD3100 or Alellix-4C (ALC-40-4C), POL3026 began to loose its activity if addition was delayed for 15 min. Furthermore, 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 HIV coreceptors.

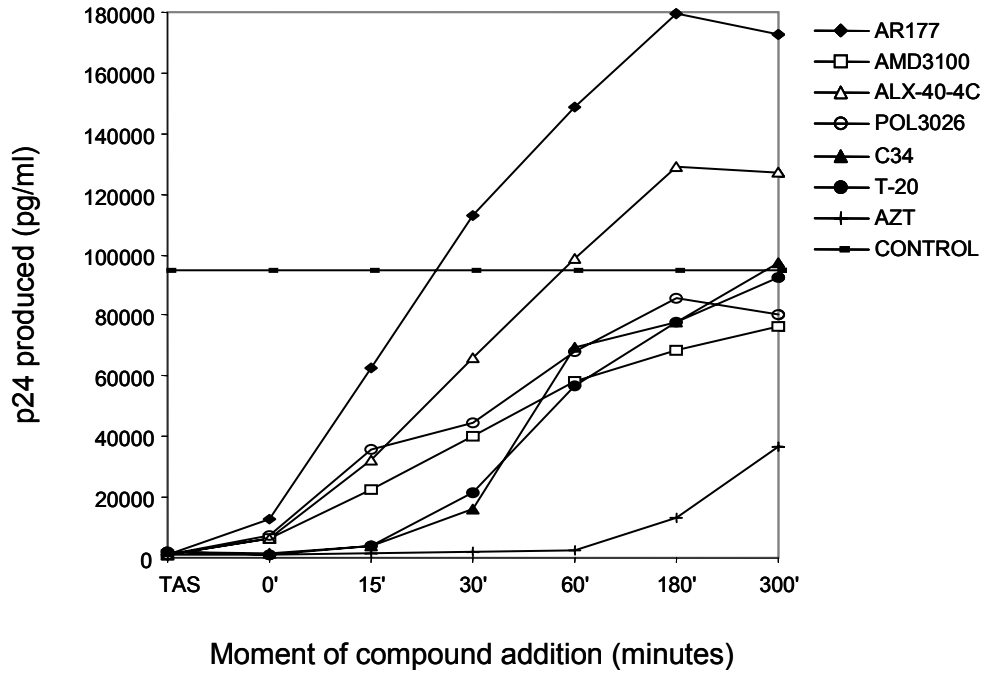


Figure 16. Time of addition experiment. Effect of the addition of compounds (binding inhibitor AR177; CXCR4 antagonists AMD3100 and ALX-40-4C; POL3026; fusion inhibitors C34 and T-20; and RT inhibitor AZT) at different moments after initiation of infection on the p24 production by NL4-3 in MT-4 cells 30 h after infection. The compounds were used at a blocking concentration of HIV replication (AR177, 50 µg/ml; AMD3100, ALX-40-4C, POL3026, C34, 1 µg/ml; T-20, 5 µg/ml; AZT, 0,5 µg/ml). TAS, temperature-arrested state. A representative time of addition experiment is shown. Similar results were obtained in four separate experiments.

POL3026 inhibits 12G5 mAb staining and CXCL12-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 (Fig. 17A) in a dose-dependent manner (IC_{50} of 0.0005 μg/ml) (Fig. 17B). Conversely, POL3026 did not interfere with the specific mAb staining of 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 a masking of the epitope recognized by the 12G5 antibody, we calculated the 50% inhibitory concentration (IC_{50}) of POL3026, AMD3100 and CXCL12 at 37ºC (both down-regulation 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 CXCL12 (1.03 μg/ml) was different (31-fold) at 4ºC, 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 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 CXCL12 in both cell lines tested (Fig. 17C). The specificity of POL3026 was further demonstrated as it could not affect the Ca^{2+} mobilization induced by CCR5-specific chemokines CCL5, CCL3 and CCL4 (data not shown), confirming the specificity of POL3026 for CXCR4.

Furthermore, POL3026 showed a potent inhibition of the chemotactic response to CXCL12 by CXCR4+ cells (Fig 17D). Taken together, these results suggest that POL3026 does not induce down-regulation and it is a potent antagonist of CXCR4.

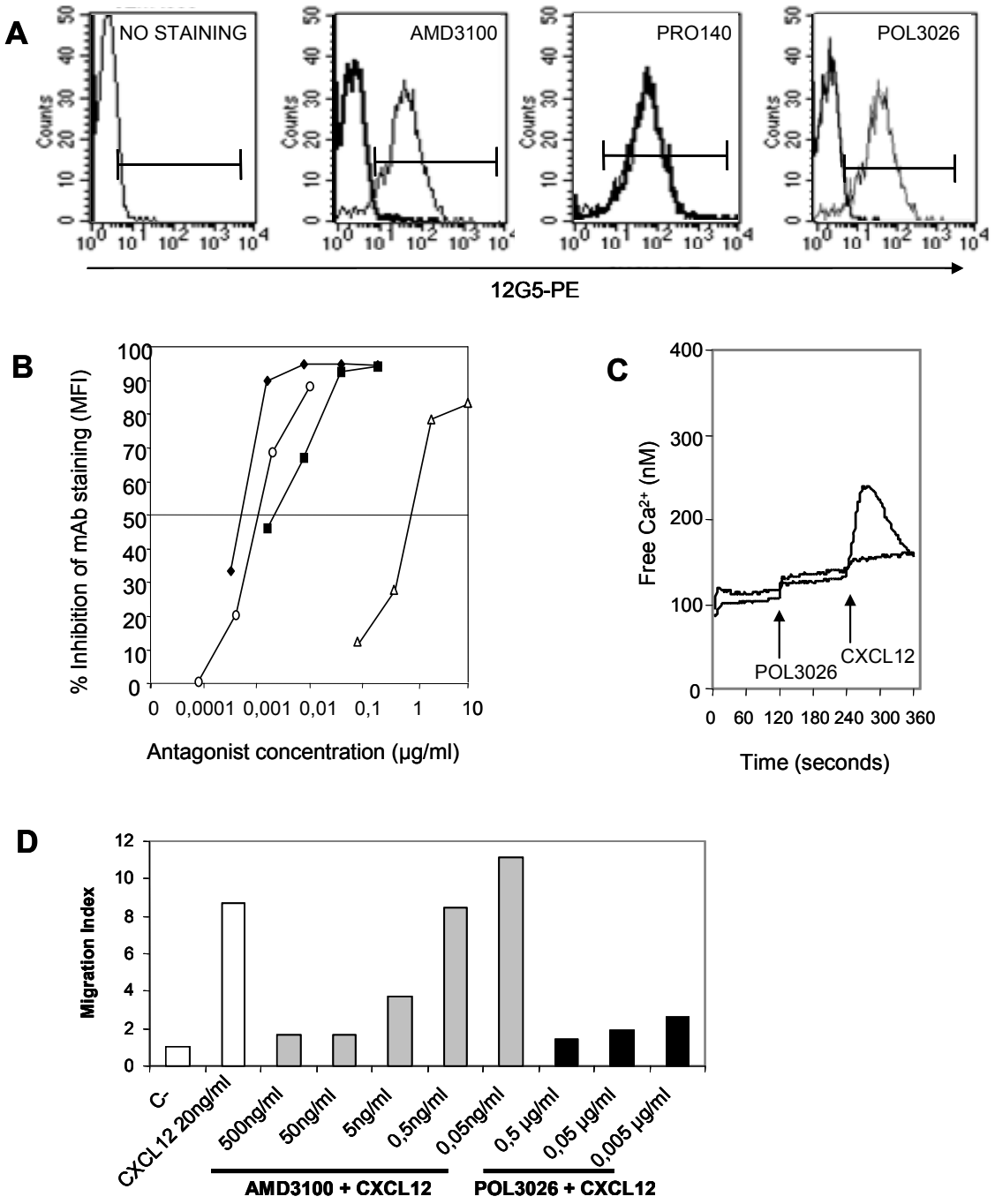


Figure 17. CXCR4 specificity and antagonism of POL3026. A, inhibition of mAb 12G5 (anti-CXCR4) staining on CEM-CCR5 cells by different compounds (CXCR4 antagonist AMD3100 at 0.2 µg/ml, anti-CCR5 mAb PRO140 at 10 µg/ml and POL3026 at 0.04 µg/ml). **B,** dose-response curve of the inhibition of staining 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 100 ng/ml of CXCL12 in $0.2x10^6$ CEM-CCR5 cells was blocked by 1 µg/ml of POL3026. Representative experiments are presented in panels A to 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 CXCL12 (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 were expressed as migration index. Data shown is representative of experiments performed at least twice.

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. 18A, 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. 18B, POL3026 blocked the replication of X4 and R5X4 strains to the same extent than 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.

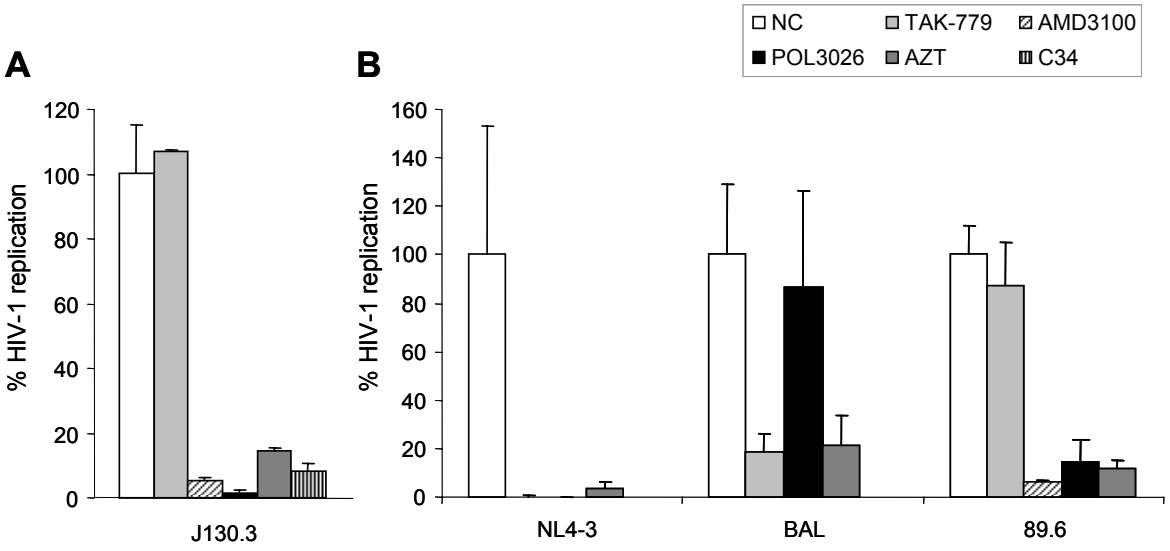


Figure 18. Anti-HIV activity of POL3026 in macrophages and lymphoid tissue cultures. A, inhibition of HIV-1 replication of J130.3 X4 strain in MDM. Concentrations of compounds used were: TAK-779 (CCR5 antagonist), 1 µg/ml; AMD3100 (CXCR4 antagonist), 5 µg/ml; POL3026, 0.008 µg/ml; AZT (RT inhibitor), 0.2 µg/ml; and C34 (fusion inhibitor), 2 µg/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 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 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 SD. NC, no compound.

Antiviral Activity of POL3026 against NL4-3 Resistant to CXCL12 and AMD3100

As shown in Table 10, POL3026 was able to block the replication of the NL4-3 strains that were made resistant to AMD3100 virus, albeit a 200-fold loss in sensitivity. Likewise, the CXCL12-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 CXCL12 in the NL4-3 backbone affect the sensitivity to POL3026, suggestive of a similar mode of action.

Table 10. Anti-HIV activity of POL3026 against NL4-3 and resistant strains

Compound	EC_{50} (μg/ml) [a] [Resistance]			CC_{50} (μg/ml) [b]
	NL4-3 wt	AMD3100res	CXCL12res	no virus
AZT	0,0006	0,0005 [0.8]	0,0006 [1]	>1
DS	0,009	0,003 [0.4]	0,005 [0.5]	>125
AMD3100	0,001	0,08 [75]	0,008 [7]	>5
C34	0,005	0,008 [2]	0,008 [2]	>5
T-20	0,1	0,01 [0.1]	0,01 [0.1]	>10
CXCL12	0,07	3 [41]	2 [30]	>25
POL3026	0,0001	0,02 [173]	0,0006 [6]	>1

[a] EC_{50}: Effective concentration 50 or needed concentration to inhibit 50% HIV-induced cell death, evaluated with the MTT method in MT-4 cells. Results shown are the mean of three separate experiments performed in triplicates. SD for all values is shown in supplementary table 2.

[b] CC_{50}: Cytotoxic concentration 50 or needed concentration to induce 50% death of non-infected cells, evaluated with the MTT method in MT-4 cells

[c] Fold Resistance: Relative loss of activity of the corresponding virus relative to the NL4-3 wt strain calculated as the Mean EC_{50} of the virus strain / Mean EC_{50} of the NL4-3 wt strain.

Development of POL3026 resistant viruses

The selection of resistant viruses to a compound can be developed through a prolonged culture of the virus with increasing concentration of this compound. Here, MT-4 cells were infected with the X4 HIV-1 strain HXB2 in the absence (HXB2.41) or presence of increasing concentrations of POL3026 for up to 205 days (41 passages in cell culture, Fig. 19A). Two virus isolates (HP41resA and HP38resB) were recovered and retitered 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. Conversely, 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 11).

Table 11. Anti-HIV activity of POL3026 against the wt, parental and resistant HXB2 strains

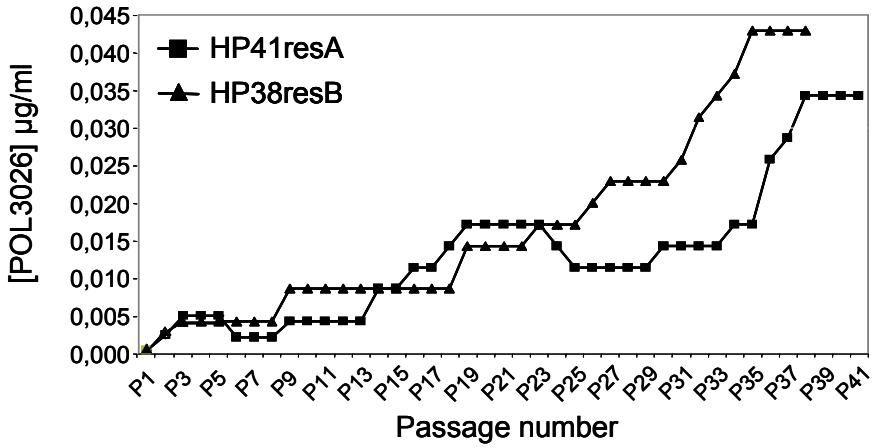
Compound	EC_{50} (µg/ml)[a] [fold-resistance][c]				CC_{50} (µg/ml)[b]
	HXB2 wild-type	HXB2 parental	HXB2 POL3026-res A	HXB2 POL3026-res B	no virus
AZT	0,001	0,001 [1]	0,001 [1]	0,001 [1]	>0,1
Nevirapine	0,04	0,03 [1]	0,03 [1]	0,08 [2]	>1
AMD3100	0,003	0,004 [1]	0,03 [10]	0,07 [22]	>1
POL3026	0,00004	0,00004 [1]	0,0005 [12]	0,001 [35]	>0,1

[a] EC_{50}: Effective concentration 50 or needed concentration to inhibit 50% HIV-induced cell death, evaluated with the MTT method in MT-4 cells. Results shown are the mean of three separate experiments performed in triplicates. SD for all values is shown in a supplementary table 2.

[b] CC_{50}: Cytotoxic concentration 50 or needed concentration to induce 50% death of non-infected cells, evaluated with the MTT method in MT-4 cells.

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

A



B

Virus	Mutations														
	C1	C2		V3					V4						C5
	96	200	232	310	316	320	325	329	396	397	398	399	400	417	473
HXB2 wt	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

C

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 - -
CXCL12r	- - - - - - E - - - - - - - - H - - - - - - - - - V - - - - - D - - - - - -
T134r	- - - - - - K - - - - - - - T K - - - - V L Y - T - T E - - D I - K - - -

Figure 19. POL3026-resistant strains. **A**, 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. **B**, mutations in gp120 that confer resistance to POL3026. Residues are numbered according to the HXB2 gp120 sequence. **C**, Comparison of V3 loop mutations that emerged under different CXCR4 targeting compounds: POL3026, AMD3100 [229], the chemokine CXCL12 [239], and the T134 [240] CXCR4 inhibitor.

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. 19B). Four mutations of these resistant viruses (Q310H, I320T, N325D, and A329T) are shared by viruses resistant to CXCL12 [239], AMD3100 [229] and T134 [240] (Fig. 19C).

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 to that of the HXB2.41 and the AMD3100-resistant virus, which has been shown to have reduced fitness [241] (Fig 20). 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.

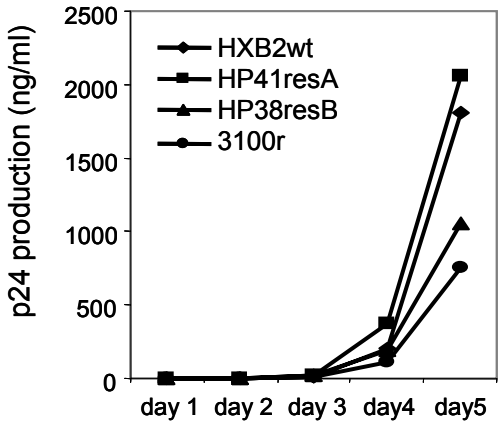


Figure 20. Growth kinetics of HXB2.41, HP41resA, HP38resB, and AMD3100-resistant virus. Virus replication was measured as p24 antigen in the supernatant of MT-4 cultures. Similar results were obtained in four separate experiments performed in triplicates.

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 (see section *HIV-1 Escape to CCR5 Coreceptor Antagonism through Selection of CXCR4-using Variants in vitro*, [242]). 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 coreceptor use to CXCR4, followed by syncytium formation and a peak in p24 viral antigen detection (Fig 21). 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).

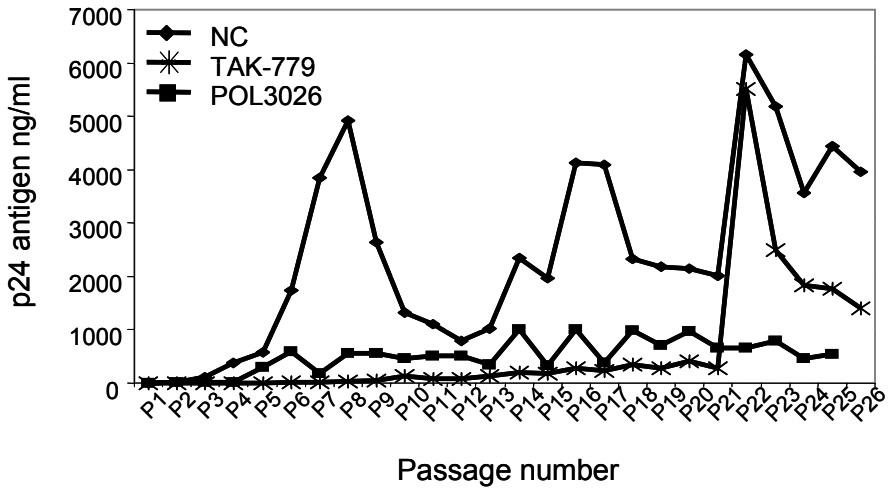


Figure 21. Growth of R5 HIV-1 168.1 strain in sustained infection of Sup-T1 cells and effect of the CCR5 antagonist TAK-779 or POL3026 on the evolution of its phenotype. Virus replication was measured as p24 antigen in the supernatant of cell culture. Low virus replication was reminiscent of R5 phenotype. Peak of p24 production reflected the gain of CXCR4 use. Representation of one of three separate experiments.

Supplementary Table 1. Anti-HIV activity of POL3026. Summary table with mean EC_{50} values represented in Tables 7 to 11

HIV Strain	Sub-type	Cells	EC_{50} (µg/ml)								
			Compound								
			POL3026	AMD3100	CXCL12	TAK-779	C34	T-20	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	-	-
CXCL12res	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	-	-
CI6	B	PBMC	0,03	0,05	-	>0,2	-	0,2	0,001	-	-
CI7	B	PBMC	0,002	0,04	-	>0,2	-	0,2	0,001	-	-
CI8	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	0,004	-	-	-	-	0,001	0,03	-
HP41resA	B	MT-4	0,0005	0,03	-	-	-	-	0,001	0,03	-
HP38resB	B	MT-4	0,001	0,07	-	-	-	-	0,001	0,08	-
CC_{50}			>1	>5	>1	>0.2	>5	>5	>1	>2	>0.1

Supplementary Table 2. Standard deviation of each mean EC_{50} value represented in Supplementary Table 1

HIV Strain	Sub-type	Cells	Standard Deviation (µg/ml)								
			Compound								
			POL3026	AMD3100	CXCL12	TAK-779	C34	T-20	AZT	Nevirapine	Efavirenz
NL4-3 wt	B	MT-4	0,00002	0,0004	0,02	-	0,002	0,1	0,0002	0,000002	0,000002
K103N	B	MT-4	0,00002	0,0001	-	-	-	-	0,0001	0,002	0,0003
IRLL98	B	MT-4	0,000002	0,00008	-	-	-	-	0,0002	-	-
Y181C	B	MT-4	0,0001	0,001	-	-	-	-	0,0001	0,4	0,0003
Y188L	B	MT-4	0,0003	0,0004	-	-	-	-	0,0003	-	-
CI-1-SI	B	MT-4	0,00008	0,002	-	-	-	-	0,2	0,002	0,00004
T20r/C34r	B	MT-4	0,0004	0,001	0,1	-	0,2	0,6	0,0003	-	-
AMD3100res	B	MT-4	0,02	0,04	2	-	0,007	0,003	0,0003	-	-
CXCL12res	B	MT-4	0,0002	0,004	1	-	0,005	0,01	0,0003	-	-
92BR014	B	MT-4	0,000002	0,002	-	-	0,000001	0,003	0,001	0,01	-
UG92029	A	MT-4	0,0001	0,003	-	-	0,00005	0,008	0,0001	0,05	-
39RW024	A	MT-4	0,000004	0,0001	-	-	0,0006	0,0004	0,0001	0,008	-
92UG021	D	MT-4	0,00001	0,0008	-	-	0,006	0,002	0,0008	0,01	-
92UG024	D	MT-4	0,0001	0,003	-	-	0,003	0,004	0,0004	0,04	-
93BR020	F	MT-4	0,00002	0,003	-	-	0,01	0,005	0,0001	0,03	-
BCF06	O	MT-4	0,00003	0,002	-	-	0,0001	0,00001	0,0002	0,002	-
ROD (HIV-2)		MT-4	0,0001	0,002	-	-	-	-	0,002	-	-
NL4-3 wt	B	PBMC	0,002	0,0008	-	-	-	0,06	0,0005	-	-
BaL	B	PBMC	-	-	-	0,006	-	0,003	0,0001	-	-
89.6	B	PBMC	0,002	0,0019	-	-	-	0,3	0,0002	-	-
CI6	B	PBMC	0,003	0,0284	-	-	-	0,07	0,0006	-	-
CI7	B	PBMC	0,0009	0,0088	-	-	-	0,1	0,0003	-	-
CI8	B	PBMC	0,0005	0,0033	-	-	-	0,07	0,00007	-	-
HXB2 wt	B	MT-4	0,00002	0,009	-	-	-	-	0,0007	-	-
HXB2.41	B	MT-4	0,00002	0,002	-	-	-	-	0,0001	-	-
HP41resA	B	MT-4	0,0001	0,007	-	-	-	-	0,00002	-	-
HP38resB	B	MT-4	0,001	0,01	-	-	-	-	0,0003	-	-

DISCUSSION

Current antiviral therapy has improved life quality of HIV+ infected individuals by reducing morbidity and mortality. However, it cannot eradicate the virus, presents long-term toxicity and is limited by the emergence of drug-resistant strains. For these reasons there is a continuous effort to design and develop new antiviral compounds targeting different steps of the HIV cycle. One interesting approach is to inhibit the steps of HIV entry to the cell and here, in this work, we focused on some of these strategies.

A study by del Real [32] in 2004 suggested that statins could inhibit HIV *in vitro* and even showed anti-HIV activity *in vivo*. For this reason we decided to evaluate statins as antiviral compounds against HIV-1. Statins are potent inhibitors of the conversion of HMG–CoA to mevalonate [191] and this leads to reduced synthesis of cholesterol and decreased prenylation of proteins [243,244]. Therefore, statins inhibit the biosynthesis of isoprenoids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate that are required for important cellular functions [245], such as the assembly of glycoproteins, heme groups and the GTP-binding proteins [246] and for the regulation of cell proliferation [247]. Previous data suggested that the anti-HIV effect of statins might be due to the inhibition of isoprenoid biosynthesis; consequently, Rho GTPase could not be prenylated at their C-terminus and remain functional. This would inhibit the rearrangement of actine cytoskeleton needed for HIV entry and budding. However, in a study of the differential inhibitory effect of lovastatin on protein isoprenylation and sterol synthesis, it was found that inhibition of protein prenylation by lovastatin could be responsible for cellular toxicity [243]. Unfortunately, we found that in standard cell culture testing and unlike control compounds (AZT, AMD3100 and DS), none of the statins had significant antiviral activity at subtoxic concentrations. We concluded that the anti-HIV activity of atorvastatin, lovastatin, simvastatin and fluvastatin may be easily confounded by their cytotoxicity in cell culture at concentrations that would be required to block HIV replication. Thus, our results suggest that concentrations required to block HIV replication *in vivo* could induce significant cytotoxicity and unwanted effects.

Nevertheless, statins are generally well tolerated and are commonly prescribed for the effective control of plasma cholesterol levels. We took advantage of a well-controlled cohort of HIV+ individuals that interrupted the antiretroviral therapy at least 3 months to evaluate the anti-HIV activity of simvastatin at a dose (80 mg daily) that showed efficacy in controlling cholesterol levels. Although there was a significant

reduction of total cholesterol and LDL-cholesterol levels in plasma, neither the mean VL or CD4+ T cell count significantly changed in all but one patient. Conversely, the former study by del Real [32], in which 6 individuals received lovastatin (40 mg daily), showed in 50 % of the patients a decrease > 0.5 log in HIV RNA in plasma as well as an increase in CD4+ T cells. We have observed similar changes in plasma HIV RNA that may occur spontaneously in HIV infected individuals not receiving any antiretroviral therapy or a lipid-lowering agent. Although differences in activity for specific statins could be claimed, our cell culture data argues against any possible effect of all these compounds, at least with the dosage and exposition lengths used in our trial.

At the same time of our study, several groups reported preliminary data on the failure of statins to control HIV replication *in vivo*. In one of these studies in HIV+ patients receiving antiretroviral treatment, pravastatin did not have any antiviral effect [248]. Also, in HIV+ patients receiving HAART no differences were detected on viral load suppression between the statin and non-statin receiving groups [249]. In another study they observed that long-term treatment with statins (pravastatin, atorvastatin or rosuvastatin) did not affect CD4+ T cell counts in HIV+ patients receiving HAART [250]. Finally, in another pilot study from our same center, HIV+ patients with viral suppression after the interruption of HAART received atorvastatin 40 or 80 mg daily, but statins failed to reduce viral rebound and CD4+ T cell loss after 4 and 12 weeks. In contradiction, basal cholesterol, but not atorvastatin influenced the viral rebound at week 4 [251]. It has been described that membrane cholesterol and lipid rafts have a role in HIV entry, budding and HIV structure and infectivity *in vitro* (reviewed in [31]). Also SP01A (http://www.samaritanpharma.com/aids_hiv_program_sp-01a.asp), a compound that reduces intracellular cholesterol levels and corticosteroid biosynthesis, showed anti-HIV activity in clinical trials. Nevertheless, the relevance of cholesterol *in vivo* and the role of lowering it by statins remains uncertain.

The failure of statins may be explained by the multiple potential effects of these compounds that are not always in an inhibitory way. The antiviral effects of statins could be compensated by an increase of HIV transcription that has been observed *in vitro* [1] or by an inhibition of the immune response [199,200]. Also, a low systemic bioavailability of statins [252], but not in the liver, could explain why a notable effect (whether favorable or not) was not observed during the 8 weeks of treatment, whereas a decrease in plasma cholesterol levels was detected. The liver is the target organ for the

statins, since it is the major site of cholesterol biosynthesis, lipoprotein production and LDL catabolism. However, extrahepatic synthesis of cholesterol is necessary for normal cell function [253]. Taken together, our results suggest that significantly higher doses may be required and this, in turn, could induce a cytotoxic, unwanted effects in HIV+ patients. Nevertheless, we cannot exclude that higher doses of simvastatin or any other statins would be effective in controlling HIV replication in humans.

Despite the fact that statins are widely used as a treatment for hyperlipidemia, their prolonged use has well-known secondary effects [254], such as myopathy [255], procarcinogenesis potential, hepatotoxicity, nerve damage [256], short temper [257], cognitive decline, memory loss [258] and teratogenic potential [259]. Moreover, there is a risk of rhabdomyolysis [260] with the concomitant use of statins in patients receiving highly active antiretroviral therapy.

In conclusion, statins represent a well-established class of drugs that effectively lower serum cholesterol levels and are widely prescribed for the treatment of hypercholesterolemia. The fact that its basic pharmacology is well-known, the study of those compounds offers perspectives at short-term in HIV treatment. However, in spite of recent studies suggesting that statins could be appropriate drugs for the treatment of HIV infection, our results, together with other *in vivo* studies, caution on the use of statins as antiretroviral agents. Further *in vivo* studies taking into account other clinical settings to try to clarify statins pleiotropic effects and determine the role of cholesterol in HIV replication *in vivo* are still needed.

Another interesting approach that has been recently developed are coreceptor inhibitors, being CCR5 antagonists the most advanced. They have shown high potency *in vitro* against R5 viruses [136-140] and proved to be effective in short-term clinical trials [141-144]. The most successful candidate was maraviroc that was recently approved by the FDA for treatment-experienced HIV+ individuals. One intriguing question is if resistance to these compounds may be developed through coreceptor switch. Emergence and selection of CXCR4 using viruses is a major concern. Nevertheless, a number of publications have suggested that CCR5 drug resistance may emerge in the absence of coreceptor switch [164,165,206-208]. HIV-1 may become resistant to vicriviroc (another CCR5 antagonist) [164,209] or maraviroc [206] *in vitro* by utilizing an inhibitor-bound form of the receptor and this has been shown as a preferential mode to circumvent the anti-HIV activity of CCR5 drugs in the absence of

coreceptor switch. Coreceptor phenotype testing from the phase II maraviroc trial showed that circulating virus remained CCR5 tropic in 60/62 patients, indicating that X4 variants were not rapidly selected despite CCR5-specific drug pressure [205]. Conversely, we showed that in cell culture, HIV-1 strains may switch faster to CXCR4 use with selective pressure on CCR5 use (TAK-779, CCL5 or 2D7 mAb) than with AZT, suggesting a preferential selection of R5X4 or X4 virus as a mode of drug resistance in some of the HIV strains tested.

The use of CCR5-targeting drugs requires the prior knowledge of the viral tropism in a given patient. Determination for HIV-1 coreceptor usage is complex and only few methods exist [215,219] that may not be sensitive enough to detect minor X4 or R5/X4 populations [95,205,215,219,261,262], which selection would be favored by a CCR5 antagonist. For instance, X4 variants in two patients on treatment with maraviroc appeared to emerge by outgrowth of a pre-treatment CXCR4-using reservoir [205].

Our observation that a clonal R5 virus may gain CXCR4 use, which also occurred in the presence of CCR5 compounds, shows that minor X4 populations may evolve from the mutants generated within the first two weeks from a purely R5 virus. Furthermore, if a minor X4 population is present at the initiation of the cell culture, it is intuitive to think that X4 emergence will occur at a similar time/rate in the absence or presence of CCR5 drugs and this did not occur. It is also relevant to bear in mind that for any given compound, the selection of a resistant virus applies for a pre-existing minor mutant population with a selective advantage in the presence of the drug. Generation of the mutant virus depends on the intrinsic mutation rate and the replicative capacity of the virus. Therefore, whether the drug-resistant virus (i.e. a CXCR4-using strain) was present at day zero or was generated during cell culture, the emergence of the mutant is independent of the drug that, if present, selects for the drug-resistant virus.

Our results are in line with a recent report of the maraviroc phase III trial concluding that more patients on maraviroc had a change in tropism to D/M or X4 at time of failure than in the placebo control group [211], underscoring the propensity of CXCR4-using virus to emerge under CCR5 drug pressure. Upon discontinuation of treatment, R5 virus may repopulate and conform the dominating phenotype, suggesting that X4 variants may only have an increased viral fitness in the presence of CCR5 drug pressure. Interestingly, in this study there was no evidence of a detrimental effect on the VL or CD4+ T cell counts due to the emergence of CXCR4-using viruses.

The X4 variants that emerged in our cultures gained sensitivity to AMD3100 and lost it to TAK-779. However, changes in susceptibility to both compounds were partial, reflecting the retained capacity of the virus to use CCR5. Notably, one virus strain expanded coreceptor capacity while retaining total sensitivity to TAK-779 in PBMC. Coreceptor switch intermediates with lower affinity to CCR5 and higher sensitivity to CCR5 inhibitors have been recently described [90]. These results were generated in U87-CD4 cells that exclusively express CCR5, allowing to evaluate changes in sensitivity due to changes in the affinity of the virus for the receptor. Reduced or loss of sensitivity to TAK-779 of HIV-1 stains in PBMC cultures, that are expressing both CCR5 and CXCR4, likely reflects increased representation of CXCR4-using variants within the virus population that outgrow in the presence of drug, and not a correlative measurement of CCR5 binding. Dualtropic viruses exhibit considerable variations in their efficiency to use CXCR4 and CCR5 as coreceptor [263], and consequently, in their susceptibility to CXCR4 and CCR5 entry inhibitors. HIV-1 strains such as 89.6, defined as dualtropic through coreceptor assays, may be completely blocked by AMD3100 in PBMC and *ex vivo* lymphoid tissue [238]. Tests for coreceptor use and drug sensitivity in cells expressing both coreceptors [263] may not always be in agreement, highlighting the necessity of multiple determinations to clearly assess coreceptor preference by HIV-1.

Evolution towards CXCR4 usage *in vivo* and *in vitro* seems to go along multiple pathways and most R5X4/X4 variants have diverse mutation patterns, although some common features (i.e. charged amino acids at position 11/25 of the V3 loop) have been detected [221,264]. We observed that the R5 isolates gained CXCR4 use via multiple mutations in gp120. With the exception of CI5 in the presence of TAK-779, which showed two different patterns of mutations, a common mutation (G314R) was observed in independent switch variants. This mutation is uncommon in CI but some cases have been reported [74,265-267] and it has been associated with gain of CXCR4 use *in vitro* [90,237,268]. In a previous study, we showed that HIV-1 CI5 may expand its coreceptor use in cell culture and this may be prevented by CXCR4 antagonists [236]. The emergence of CXCR4 variants required 100 days rather than 10-16 days as shown herein and did not involve the G-to-R mutation at position 314 but the canonical changes at positions 11 and 25 of the V3 loop. A faster coreceptor switch may occur by passaging both infected cells and supernatant and not virus-containing supernatant

alone. It is also possible that cell to cell transmission may favor virus coreceptor switch and allow for a different pattern of mutations.

In this cell culture model not all strains were able to switch coreceptor preference, which may reflect an intrinsic capacity of some isolates to switch to X4 or retain the R5 phenotype. It is enticing to suggest the importance of evaluating a significant number of isolates in order to validate if cell culture assays as our model could be used to measure the propensity of a clinical isolate to switch or expand coreceptor preference prior to or after the initiation of a CCR5 drug-containing regimen. It will also be important to determine by clonal analysis if the emerging X4 phenotype is generated by a mixture of R5 and emerging X4 variants, or are dualtropic (R5X4) viruses.

It becomes clear that cell culture conditions and choice of virus isolate are of utmost importance to induce a coreceptor change in cell culture. The relevance of our study could be questioned, but this observation can be extended to the development of resistance to CCR5 inhibitors in the absence of coreceptor switch. The virus strains that did not switch coreceptor preference did not become resistant to TAK-779 at the time that cell cultures were stopped (CI1, CI2 and BaL, data not shown). Resistance is commonly developed in cells expressing detectable CCR5 levels and by gradually increasing the compound concentration until relatively high levels are reached [165,207,208]**.** The cell type, time in culture and the concentration of HIV inhibitors together with the specific HIV-1 strain that is being selected are factors that affect the outcome of *in vitro* resistance development. Although some studies suggest that target availability is not the driving force for the coreceptor switch, determined by coreceptor expression on T cell of PBMC [269], our results also suggest that the lack of CCR5 (very low availability) and not the CXCR4 abundance could be relevant in the emergence of CXCR4-using viruses during the course of infection. Of course, studies determining coreceptor expression and occupancy by antagonists should be performed and it is obvious that other selective pressures *in vivo* could be affecting the viral populations.

The possibility of R5X4 and X4 emergence due to CCR5 antagonism and a possible role of CXCR4 antagonists to prevent it, suggest that these compounds could be useful alone or in combination with CCR5-targeting agents. Although CXCR4 antagonists have shown anti-HIV activity in clinical trials, at present there are not any

CXCR4 antagonist candidates under clinical development (see introduction), therefore there is a need for CXCR4 targeting compounds with a safety profile suitable for human clinical use. For this reason we decided to collaborate with Polyphor biotech company who designed several CXCR4 antagonists. Previous work selected POL3026 by its plasma stability, high selectivity for CXCR4 and favorable pharmacokinetic properties in dogs [226]. In our study, we characterized 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 or chemotaxis, but it interfered with the calcium signaling and chemotaxis induced by CXCL12, suggesting that POL3026 acts as an antagonist of CXCR4. Furthermore, multiparametric evaluation of HIV envelope function and time of addition experiments suggested that POL3026 blocks HIV replication at the level that corresponds with the virus interaction with the 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) that are resistant to other CXCR4 ligands. From the above results it was not surprising the emergence and location of mutations developed under selective pressure with this compound, which appeared mainly in the V3 loop of gp120. Four mutations (Q310H, I320T, N325D, and A329T) are shared by viruses resistant to CXCL12 [239], AMD3100 [229] and T134 [240], what could explain the cross-resistance observed between AMD3100 and POL3026. Cross-resistance may not be obligatory as ligands such as AMD3100, CXCL12 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 or HXB2, cross-resistance suggests a similar mode of action. Of note, development of POL3026 resistance did not select viral strains that switched to CCR5 usage, but as disscussed before, culture circumstances during passages and the viral strain can be conditioners for viral escape through coreceptor switch. The mutations of POL3026 resistant viruses did not have a clear fitness cost as measured by virus competition assays. However, growth kinetics indicated that HP38resB, which 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 [241]. However, the number of mutations of the

AMD3100-resistant virus (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.

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 one of the most active anti-CXCR4 agents known to date. POL3026 blocked the replication of macrophage tropic HIV-1 J130.3 strain that has X4 phenotype. 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. It did not inhibit the replication of R5 tropic virus, but it was 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 [238]. However, some of clinical isolates showed a loss of sensitivity to POL3026 and AMD3100 in PBMC compared to the NL4-3 strain, probably due to their capacity to use both coreceptors. Unfortunately, in a recent study where AMD3100 was evaluated against a panel of R5X4 and X4 strains [270], a basal resistance to this compound could be measured in several viruses as a decreased maximum inhibition of HIV replication (plateau effect). The magnitude of the plateau varied also depending on the cell-donor, although the mechanisms were unknown. This baseline resistance could affect the clinical use of this class of compounds and although more results are necessary to corroborate that, it would be interesting to test POL3026 against a broader panel of CI in PBMC from different donors. Data of anti-HIV activity in PBMC showed here was evaluated in a pool of PBMC from six different donors, thus avoiding this possible host factor effect.

We also showed 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 [236] and recent studies have shown

that roughly 20% of drug-naïve (untreated) individuals may harbor X4 viruses [271,272], a percentage that increases up to 58% among drug-experienced individuals [273]. 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 [274], leading to coreceptor switch under CCR5 antagonist treatment [211]. 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. Nevertheless, antagonism to CXCR4 in long-term therapy, in contrast to CCR5 antagonism, is questioned because of the unknown consequences of inhibiting a receptor involved in important biological functions [53,59] such as hematopoietic stem cell mobilization, homing and trafficking of leukocytes, brain development, vascularization and T cell activation and migration to sites of inflammation. However, in the halted clinical trials i.e. AMD070 [173], the toxicity observed seems not to be target-related. In terms of CXCR4 coreceptor inhibitors, the ultimate strategy would be inhibiting HIV binding to CXCR4 while retaining the receptor functionality through CXCL12. In the lack of a non-agonistic non-antagonistic CXCR4 inhibitor, the option would be a compound with the highest possible anti-HIV activity / CXCR4 antagonism rate.

Concluding remarks

Can translational research truly provide a fast or immediate response about potential new therapeutic agents in order to make informed decisions on, for example, relevant treatment options in HIV infection? May we provide laboratory-based evidence that parallels what it is observed in patients? This thesis has been a practical attempt to confirm and demonstrate that we are able to do it. In a relatively short time from the first description of the potential use of statins to treat HIV infection, we provided strong support that argued against the efficacy of statins as antiretrovirals. In an attempt to model virus evolution we showed that drugs against CCR5 may curve the natural

history of HIV towards apparently more pathogenic variants. In HIV research, laboratory based evidence seems to be, as never before, closer to the clinic.

Nevertheless, results generated during this research thesis, regardless of the controversial aspects of some of them (e.g. do X4 viruses truly emerge from the R5 after CCR5 drug pressure or are they the simple consequence of selecting for X4 minor species that were already there?) leave more questions open than answered and call for basic research as the means to resolve them. Our results suggest that effective blockage of CCR5 may prompt the evolution of HIV towards CXCR4 usage; conversely, allowing CCR5 use may prompt CCR5-drug resistance while maintaining the R5 phenotype. Our results may pose a paradox: it is the drug efficacy, that is CCR5 occupancy, that leads to the unwanted coreceptor switch. On the other hand, suboptimal (<100% receptor occupancy) concentrations of drug may be the cause of drug-resistance without switch. A current paradigm of antiretroviral therapy tells that complete drug efficacy is the way to avoid resistance. We may have laboratory evidence to prove that for coreceptor-based drugs this may be too simplistic. Again, would it be posible to show in the patients what we observed in the lab? We will require a firm hypothesis to convince that coreceptor occupancy *in vivo* may be the key to unraveling when and how CCR5 drug resistance occurs. While we may simply measure drug and coreceptor levels in blood and possible in secondary lymphoid tissue, virus compartments such as the gut or the CNS may not be readily available for testing and evaluation.

Another question that was only superficially looked at during the years of this thesis research was CXCR4 and its role in HIV infection. Much has said and we only provided evidence of how more potent anti-HIV agents can be generated. But CXCR4 represents a tremendous obstacle. Can we develope a strategy to target X4 variants without unwanted CXCR4-dependent effects? It will be up to other to continue these lines of research and provide evidence-based answers. I hope our work may have contributed to find them and to better understand the role of coreceptors in HIV therapy and infection, opening new avenues for future research.

CONCLUSIONS

- According to standard cell culture testing, statins do not have significant anti-HIV activity and in previous observations it may have been confounded by their accrued cytotoxicity at concentrations that would be required to block HIV activity. Simvastatin showed no effect on viral load or CD4+ T cell counts in 12 HIV+ patients, supporting the *in vitro* data. Our results caution on the use of statins as antiretroviral agents.

- An *in vitro* model that we used to study coreceptor switch of R5 HIV-1 strains to R5X4 or X4 permitted us to conclude that the probability to change coreceptor use depends on the R5 isolate and the cell-culture conditions, being the CCR5 expression of utmost relevance. Therefore, this model could be interesting to asses the tendency of HIV clinical isolates to develop resistance to CCR5 agents through a switch in coreceptor usage.

- The selective pressure of an anti-HIV compound can modify the evolution of coreceptor usage. Reverse transcriptase inhibitors and CCR5 targeting agents delayed the emergence of CXCR4-using variants compared to untreated cultures. However, CXCR4-using variants emerged faster under CCR5 drug pressure than under reverse transcriptase inhibitors, whereas CXCR4 antagonists could prevent the emergence of CXCR4-using variants. Our results suggest that HIV-1 strains that are prone to switch coreceptor may escape CCR5 drug pressure through selecting CXCR4-using variants, whereas compounds targeting CXCR4 prevent the emergence of R5X4 or X4 strains.

- POL3026 is a potent anti-HIV agent against naïve and drug resistant clinical isolates with X4 and R5X4 phenotype. POL3026 blocks HIV replication through specific interaction with CXCR4 and it acts as an antagonist of this chemokine receptor. Study of pattern mutations selected in the resistant viruses proved to be a useful tool to confirm its mode of action. Development of resistance to POL3026 slightly affect the replication of the viruses respect to the wild-type and it did not induce a coreceptor switch in the conditions used. POL3026 with its high potency and specificity may represent an step forward in the design of a prototype CXCR4 inhibitor that will continue further development.

REFERENCES

1. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, et al. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220: 868-871.
2. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, et al. (1984) Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224: 500-503.
3. Popovic M, Sarngadharan MG, Read E, Gallo RC (1984) Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224: 497-500.
4. Embretson J, Zupancic M, Ribas JL, Burke A, Racz P, et al. (1993) Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature 362: 359-362.
5. Rambaut A, Posada D, Crandall KA, Holmes EC (2004) The causes and consequences of HIV evolution. Nat Rev Genet 5: 52-61.
6. Coffin, editor (1992) Structure and classification of retroviruses. New York: In J. Levy (ed.), Plenum Press. vol 1, p19-50 p.
7. Briggs JA, Wilk T, Welker R, Krausslich HG, Fuller SD (2003) Structural organization of authentic, mature HIV-1 virions and cores. Embo J 22: 1707-1715.
8. Zhu P, Liu J, Bess J, Jr., Chertova E, Lifson JD, et al. (2006) Distribution and three-dimensional structure of AIDS virus envelope spikes. Nature 441: 847-852.
9. Muesing MA, Smith DH, Cabradilla CD, Benton CV, Lasky LA, et al. (1985) Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature 313: 450-458.
10. Frankel AD, Young JA (1998) HIV-1: fifteen proteins and an RNA. Annu Rev Biochem 67: 1-25.
11. Steffens CM, Hope TJ (2001) Recent advances in the understanding of HIV accessory protein function. Aids 15 Suppl 5: S21-26.
12. Liang C, Wainberg MA (2002) The role of Tat in HIV-1 replication: an activator and/or a suppressor? AIDS Rev 4: 41-49.
13. Goff SP (2003) Death by deamination: a novel host restriction system for HIV-1. Cell 114: 281-283.

14. Bukrinsky MI, Sharova N, Dempsey MP, Stanwick TL, Bukrinskaya AG, et al. (1992) Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc Natl Acad Sci U S A 89: 6580-6584.

15. Gallay P, Swingler S, Song J, Bushman F, Trono D (1995) HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. Cell 83: 569-576.

16. Scondras D (2007) Is cure possible for HIV? AIDS Treat News: 6-7.

17. Gondois-Rey F, Biancotto A, Pion M, Chenine AL, Gluschankof P, et al. (2001) Production of HIV-1 by resting memory T lymphocytes. Aids 15: 1931-1940.

18. Neumann M, Harrison J, Saltarelli M, Hadziyannis E, Erfle V, et al. (1994) Splicing variability in HIV type 1 revealed by quantitative RNA polymerase chain reaction. AIDS Res Hum Retroviruses 10: 1531-1542.

19. Cosson P (1996) Direct interaction between the envelope and matrix proteins of HIV-1. Embo J 15: 5783-5788.

20. Kohl NE, Emini EA, Schleif WA, Davis LJ, Heimbach JC, et al. (1988) Active human immunodeficiency virus protease is required for viral infectivity. Proc Natl Acad Sci U S A 85: 4686-4690.

21. Berger EA, Murphy PM, Farber JM (1999) Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu Rev Immunol 17: 657-700.

22. Clark SJ, Saag MS, Decker WD, Campbell-Hill S, Roberson JL, et al. (1991) High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. N Engl J Med 324: 954-960.

23. Lyles RH, Munoz A, Yamashita TE, Bazmi H, Detels R, et al. (2000) Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study. J Infect Dis 181: 872-880.

24. Mellors JW, Kingsley LA, Rinaldo CR, Jr., Todd JA, Hoo BS, et al. (1995) Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. Ann Intern Med 122: 573-579.

25. McCune JM (2001) The dynamics of CD4+ T-cell depletion in HIV disease. Nature 410: 974-979.

26. Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, et al. (1995) Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373: 117-122.

27. Cao Y, Qin L, Zhang L, Safrit J, Ho DD (1995) Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. N Engl J Med 332: 201-208.

28. Este JA, Telenti A (2007) HIV entry inhibitors. Lancet 370: 81-88.

29. Ugolini S, Mondor I, Sattentau QJ (1999) HIV-1 attachment: another look. Trends Microbiol 7: 144-149.

30. Gallo SA, Finnegan CM, Viard M, Raviv Y, Dimitrov A, et al. (2003) The HIV Env-mediated fusion reaction. Biochim Biophys Acta 1614: 36-50.

31. Campbell SM, Crowe SM, Mak J (2001) Lipid rafts and HIV-1: from viral entry to assembly of progeny virions. J Clin Virol 22: 217-227.

32. del Real G, Jimenez-Baranda S, Mira E, Lacalle RA, Lucas P, et al. (2004) Statins inhibit HIV-1 infection by down-regulating Rho activity. J Exp Med 200: 541-547.

33. Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, et al. (1984) The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312: 763-767.

34. Feng Y, Broder CC, Kennedy PE, Berger EA (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272: 872-877.

35. Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, et al. (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 381: 667-673.

36. Miceli MC, Parnes JR (1993) Role of CD4 and CD8 in T cell activation and differentiation. Adv Immunol 53: 59-122.

37. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, et al. (1998) Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393: 648-659.

38. Edinger AL, Mankowski JL, Doranz BJ, Margulies BJ, Lee B, et al. (1997) CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain. Proc Natl Acad Sci U S A 94: 14742-14747.

39. Dumonceaux J, Nisole S, Chanel C, Quivet L, Amara A, et al. (1998) Spontaneous mutations in the env gene of the human immunodeficiency virus type 1 NDK isolate are associated with a CD4-independent entry phenotype. J Virol 72: 512-519.

40. LaBranche CC, Hoffman TL, Romano J, Haggarty BS, Edwards TG, et al. (1999) Determinants of CD4 independence for a human immunodeficiency virus type 1 variant map outside regions required for coreceptor specificity. J Virol 73: 10310-10319.

41. Endres MJ, Clapham PR, Marsh M, Ahuja M, Turner JD, et al. (1996) CD4-independent infection by HIV-2 is mediated by fusin/CXCR4. Cell 87: 745-756.

42. Oppermann M (2004) Chemokine receptor CCR5: insights into structure, function, and regulation. Cell Signal 16: 1201-1210.

43. Zhao J, Ma L, Wu YL, Wang P, Hu W, et al. (1998) Chemokine receptor CCR5 functionally couples to inhibitory G proteins and undergoes desensitization. J Cell Biochem 71: 36-45.

44. Signoret N, Christophe T, Oppermann M, Marsh M (2004) pH-independent endocytic cycling of the chemokine receptor CCR5. Traffic 5: 529-543.

45. Doms RW (2001) Chemokine receptors and HIV entry. Aids 15 Suppl 1: S34-35.

46. Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, et al. (1996) A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell 85: 1149-1158.

47. Horuk R, Hesselgesser J, Zhou Y, Faulds D, Halks-Miller M, et al. (1998) The CC chemokine I-309 inhibits CCR8-dependent infection by diverse HIV-1 strains. J Biol Chem 273: 386-391.

48. Blanpain C, Migeotte I, Lee B, Vakili J, Doranz BJ, et al. (1999) CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. Blood 94: 1899-1905.

49. Ruffing N, Sullivan N, Sharmeen L, Sodroski J, Wu L (1998) CCR5 has an expanded ligand-binding repertoire and is the primary receptor used by MCP-2 on activated T cells. Cell Immunol 189: 160-168.

50. Samson M, Labbe O, Mollereau C, Vassart G, Parmentier M (1996) Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochemistry 35: 3362-3367.

51. Wong MM, Fish EN (2003) Chemokines: attractive mediators of the immune response. Semin Immunol 15: 5-14.

52. Luther SA, Cyster JG (2001) Chemokines as regulators of T cell differentiation. Nat Immunol 2: 102-107.

53. Murdoch C, Finn A (2000) Chemokine receptors and their role in inflammation and infectious diseases. Blood 95: 3032-3043.

54. Doranz BJ, Lu ZH, Rucker J, Zhang TY, Sharron M, et al. (1997) Two distinct CCR5 domains can mediate coreceptor usage by human immunodeficiency virus type 1. J Virol 71: 6305-6314.

55. Dragic T (2001) An overview of the determinants of CCR5 and CXCR4 co-receptor function. J Gen Virol 82: 1807-1814.

56. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, et al. (1995) Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science 270: 1811-1815.

57. Huang Y, Paxton WA, Wolinsky SM, Neumann AU, Zhang L, et al. (1996) The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat Med 2: 1240-1243.

58. Lim JK, Louie CY, Glaser C, Jean C, Johnson B, et al. (2008) Genetic deficiency of chemokine receptor CCR5 is a strong risk factor for symptomatic West Nile virus infection: a meta-analysis of 4 cohorts in the US epidemic. J Infect Dis 197: 262-265.

59. Kucia M, Reca R, Miekus K, Wanzeck J, Wojakowski W, et al. (2005) Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. Stem Cells 23: 879-894.

60. Burger JA, Kipps TJ (2006) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107: 1761-1767.

61. Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, et al. (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature 382: 829-833.

62. Brelot A, Heveker N, Montes M, Alizon M (2000) Identification of residues of CXCR4 critical for human immunodeficiency virus coreceptor and chemokine receptor activities. J Biol Chem 275: 23736-23744.

63. Tian S, Choi WT, Liu D, Pesavento J, Wang Y, et al. (2005) Distinct functional sites for human immunodeficiency virus type 1 and stromal cell-derived factor 1alpha on CXCR4 transmembrane helical domains. J Virol 79: 12667-12673.

64. Chen B, Vogan EM, Gong H, Skehel JJ, Wiley DC, et al. (2005) Structure of an unliganded simian immunodeficiency virus gp120 core. Nature 433: 834-841.

65. Liu J, Bartesaghi A, Borgnia MJ, Sapiro G, Subramaniam S (2008) Molecular architecture of native HIV-1 gp120 trimers. Nature 455: 109-113.

66. Allan JS, Coligan JE, Barin F, McLane MF, Sodroski JG, et al. (1985) Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. Science 228: 1091-1094.

67. Starcich BR, Hahn BH, Shaw GM, McNeely PD, Modrow S, et al. (1986) Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell 45: 637-648.

68. Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, et al. (1990) Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J Biol Chem 265: 10373-10382.

69. Wyatt R, Kwong PD, Desjardins E, Sweet RW, Robinson J, et al. (1998) The antigenic structure of the HIV gp120 envelope glycoprotein. Nature 393: 705-711.

70. Capon DJ, Ward RH (1991) The CD4-gp120 interaction and AIDS pathogenesis. Annu Rev Immunol 9: 649-678.

71. Ohgimoto S, Shioda T, Mori K, Nakayama EE, Hu H, et al. (1998) Location-specific, unequal contribution of the N glycans in simian immunodeficiency virus gp120 to viral infectivity and removal of multiple glycans without disturbing infectivity. J Virol 72: 8365-8370.

72. Kowalski M, Potz J, Basiripour L, Dorfman T, Goh WC, et al. (1987) Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science 237: 1351-1355.

73. Menendez-Arias L, Este JA (2004) HIV-resistance to viral entry inhibitors. Curr Pharm Des 10: 1845-1860.

74. LaRosa GJ, Davide JP, Weinhold K, Waterbury JA, Profy AT, et al. (1990) Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. Science 249: 932-935.

75. Milich L, Margolin B, Swanstrom R (1993) V3 loop of the human immunodeficiency virus type 1 Env protein: interpreting sequence variability. J Virol 67: 5623-5634.

76. Huang CC, Tang M, Zhang MY, Majeed S, Montabana E, et al. (2005) Structure of a V3-containing HIV-1 gp120 core. Science 310: 1025-1028.

77. Stanfield R, Cabezas E, Satterthwait A, Stura E, Profy A, et al. (1999) Dual conformations for the HIV-1 gp120 V3 loop in complexes with different neutralizing fabs. Structure 7: 131-142.

78. Sander O, Sing T, Sommer I, Low AJ, Cheung PK, et al. (2007) Structural descriptors of gp120 V3 loop for the prediction of HIV-1 coreceptor usage. PLoS Comput Biol 3: e58.

79. Rizzuto CD, Wyatt R, Hernandez-Ramos N, Sun Y, Kwong PD, et al. (1998) A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. Science 280: 1949-1953.

80. de Jong JJ, Goudsmit J, Keulen W, Klaver B, Krone W, et al. (1992) Human immunodeficiency virus type 1 clones chimeric for the envelope V3 domain differ in syncytium formation and replication capacity. J Virol 66: 757-765.

81. Fouchier RA, Groenink M, Kootstra NA, Tersmette M, Huisman HG, et al. (1992) Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. J Virol 66: 3183-3187.

82. Kwong PD (2005) Human immunodeficiency virus: refolding the envelope. Nature 433: 815-816.

83. Judice JK, Tom JY, Huang W, Wrin T, Vennari J, et al. (1997) Inhibition of HIV type 1 infectivity by constrained alpha-helical peptides: implications for the viral fusion mechanism. Proc Natl Acad Sci U S A 94: 13426-13430.

84. Jiang S, Lin K, Strick N, Neurath AR (1993) Inhibition of HIV-1 infection by a fusion domain binding peptide from the HIV-1 envelope glycoprotein GP41. Biochem Biophys Res Commun 195: 533-538.

85. Kilby JM, Hopkins S, Venetta TM, DiMassimo B, Cloud GA, et al. (1998) Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. Nat Med 4: 1302-1307.

86. Tan K, Liu J, Wang J, Shen S, Lu M (1997) Atomic structure of a thermostable subdomain of HIV-1 gp41. Proc Natl Acad Sci U S A 94: 12303-12308.

87. Chan DC, Fass D, Berger JM, Kim PS (1997) Core structure of gp41 from the HIV envelope glycoprotein. Cell 89: 263-273.

88. Berger EA, Doms RW, Fenyo EM, Korber BT, Littman DR, et al. (1998) A new classification for HIV-1. Nature 391: 240.

89. Pollakis G, Kang S, Kliphuis A, Chalaby MI, Goudsmit J, et al. (2001) N-linked glycosylation of the HIV type-1 gp120 envelope glycoprotein as a major determinant of CCR5 and CXCR4 coreceptor utilization. J Biol Chem 276: 13433-13441.

90. Pastore C, Nedellec R, Ramos A, Hartley O, Miamidian JL, et al. (2007) Conserved changes in envelope function during human immunodeficiency virus type 1 coreceptor switching. J Virol 81: 8165-8179.

91. Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR (1997) Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals. J Exp Med 185: 621-628.

92. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, et al. (1996) Identification of a major co-receptor for primary isolates of HIV-1. Nature 381: 661-666.

93. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, et al. (1996) Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell 86: 367-377.

94. Koot M, Keet IP, Vos AH, de Goede RE, Roos MT, et al. (1993) Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. Ann Intern Med 118: 681-688.

95. Wilkin TJ, Su Z, Kuritzkes DR, Hughes M, Flexner C, et al. (2007) HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS Clinical Trial Group A5211. Clin Infect Dis 44: 591-595.

96. Moyle GJ, Wildfire A, Mandalia S, Mayer H, Goodrich J, et al. (2005) Epidemiology and predictive factors for chemokine receptor use in HIV-1 infection. J Infect Dis 191: 866-872.

97. Brumme ZL, Goodrich J, Mayer HB, Brumme CJ, Henrick BM, et al. (2005) Molecular and clinical epidemiology of CXCR4-using HIV-1 in a large population of antiretroviral-naive individuals. J Infect Dis 192: 466-474.

98. Van Baelen K, Vandenbroucke I, Rondelez E, Van Eygen V, Vermeiren H, et al. (2007) HIV-1 coreceptor usage determination in clinical isolates using clonal and population-based genotypic and phenotypic assays. J Virol Methods 146: 61-73.

99. de Mendoza C, Van Baelen K, Poveda E, Rondelez E, Zahonero N, et al. (2008) Performance of a population-based HIV-1 tropism phenotypic assay and correlation with V3 genotypic prediction tools in recent HIV-1 seroconverters. J Acquir Immune Defic Syndr 48: 241-244.

100. Hunt PW, Harrigan PR, Huang W, Bates M, Williamson DW, et al. (2006) Prevalence of CXCR4 tropism among antiretroviral-treated HIV-1-infected patients with detectable viremia. J Infect Dis 194: 926-930.

101. Richman DD, Bozzette SA (1994) The impact of the syncytium-inducing phenotype of human immunodeficiency virus on disease progression. J Infect Dis 169: 968-974.

102. Shioda T, Levy JA, Cheng-Mayer C (1992) Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell-line and macrophage tropism of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A 89: 9434-9438.

103. Chan SY, Speck RF, Power C, Gaffen SL, Chesebro B, et al. (1999) V3 recombinants indicate a central role for CCR5 as a coreceptor in tissue infection by human immunodeficiency virus type 1. J Virol 73: 2350-2358.

104. Hwang SS, Boyle TJ, Lyerly HK, Cullen BR (1991) Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science 253: 71-74.

105. Coffin JM (1995) HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science 267: 483-489.

106. Moore JP, Kitchen SG, Pugach P, Zack JA (2004) The CCR5 and CXCR4 coreceptors--central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses 20: 111-126.

107. Pastore C, Ramos A, Mosier DE (2004) Intrinsic obstacles to human immunodeficiency virus type 1 coreceptor switching. J Virol 78: 7565-7574.

108. Bunnik EM, Quakkelaar ED, van Nuenen AC, Boeser-Nunnink B, Schuitemaker H (2007) Increased neutralization sensitivity of recently emerged CXCR4-using

human immunodeficiency virus type 1 strains compared to coexisting CCR5-using variants from the same patient. J Virol 81: 525-531.

109. Ho SH, Tasca S, Shek L, Li A, Gettie A, et al. (2007) Coreceptor switch in R5-tropic simian/human immunodeficiency virus-infected macaques. J Virol 81: 8621-8633.

110. Tersmette M, Miedema F (1990) Interactions between HIV and the host immune system in the pathogenesis of AIDS. Aids 4 Suppl 1: S57-66.

111. Ribeiro RM, Hazenberg MD, Perelson AS, Davenport MP (2006) Naive and memory cell turnover as drivers of CCR5-to-CXCR4 tropism switch in human immunodeficiency virus type 1: implications for therapy. J Virol 80: 802-809.

112. Kreisberg JF, Kwa D, Schramm B, Trautner V, Connor R, et al. (2001) Cytopathicity of human immunodeficiency virus type 1 primary isolates depends on coreceptor usage and not patient disease status. J Virol 75: 8842-8847.

113. Jekle A, Keppler OT, De Clercq E, Schols D, Weinstein M, et al. (2003) In vivo evolution of human immunodeficiency virus type 1 toward increased pathogenicity through CXCR4-mediated killing of uninfected CD4 T cells. J Virol 77: 5846-5854.

114. Shepherd JC, Jacobson LP, Qiao W, Jamieson BD, Phair JP, et al. (2008) Emergence and persistence of CXCR4-tropic HIV-1 in a population of men from the multicenter AIDS cohort study. J Infect Dis 198: 1104-1112.

115. Burger H, Hoover D (2008) HIV-1 tropism, disease progression, and clinical management. J Infect Dis 198: 1095-1097.

116. Carr A (2003) Toxicity of antiretroviral therapy and implications for drug development. Nat Rev Drug Discov 2: 624-634.

117. Stevenson M (2003) HIV-1 pathogenesis. Nat Med 9: 853-860.

118. Steigbigel RT, Cooper DA, Kumar PN, Eron JE, Schechter M, et al. (2008) Raltegravir with optimized background therapy for resistant HIV-1 infection. N Engl J Med 359: 339-354.

119. Vives RR, Imberty A, Sattentau QJ, Lortat-Jacob H (2005) Heparan sulfate targets the HIV-1 envelope glycoprotein gp120 coreceptor binding site. J Biol Chem 280: 21353-21357.

120. Este JA, Cabrera C, Schols D, Cherepanov P, Gutierrez A, et al. (1998) Human immunodeficiency virus glycoprotein gp120 as the primary target for the antiviral action of AR177 (Zintevir). Mol Pharmacol 53: 340-345.

121. Daar ES, Li XL, Moudgil T, Ho DD (1990) High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. Proc Natl Acad Sci U S A 87: 6574-6578.

122. Martin L, Stricher F, Misse D, Sironi F, Pugniere M, et al. (2003) Rational design of a CD4 mimic that inhibits HIV-1 entry and exposes cryptic neutralization epitopes. Nat Biotechnol 21: 71-76.

123. Stricher F, Martin L, Barthe P, Pogenberg V, Mechulam A, et al. (2005) A high-throughput fluorescence polarization assay specific to the CD4 binding site of HIV-1 glycoproteins based on a fluorescein-labelled CD4 mimic. Biochem J 390: 29-39.

124. Allaway GP, Davis-Bruno KL, Beaudry GA, Garcia EB, Wong EL, et al. (1995) Expression and characterization of CD4-IgG2, a novel heterotetramer that neutralizes primary HIV type 1 isolates. AIDS Res Hum Retroviruses 11: 533-539.

125. Jacobson JM, Israel RJ, Lowy I, Ostrow NA, Vassilatos LS, et al. (2004) Treatment of advanced human immunodeficiency virus type 1 disease with the viral entry inhibitor PRO 542. Antimicrob Agents Chemother 48: 423-429.

126. Lin PF, Blair W, Wang T, Spicer T, Guo Q, et al. (2003) A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. Proc Natl Acad Sci U S A 100: 11013-11018.

127. Ho HT, Fan L, Nowicka-Sans B, McAuliffe B, Li CB, et al. (2006) Envelope conformational changes induced by human immunodeficiency virus type 1 attachment inhibitors prevent CD4 binding and downstream entry events. J Virol 80: 4017-4025.

128. Guo Q, Ho HT, Dicker I, Fan L, Zhou N, et al. (2003) Biochemical and genetic characterizations of a novel human immunodeficiency virus type 1 inhibitor that blocks gp120-CD4 interactions. J Virol 77: 10528-10536.

129. (2004) Attachment inhibitor may hold promise for future. BMS-488043 demonstrates safety. AIDS Alert 19: 57-58.

130. Burkly LC, Olson D, Shapiro R, Winkler G, Rosa JJ, et al. (1992) Inhibition of HIV infection by a novel CD4 domain 2-specific monoclonal antibody. Dissecting the basis for its inhibitory effect on HIV-induced cell fusion. J Immunol 149: 1779-1787.

131. Moore JP, Sattentau QJ, Klasse PJ, Burkly LC (1992) A monoclonal antibody to CD4 domain 2 blocks soluble CD4-induced conformational changes in the envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) and HIV-1 infection of CD4+ cells. J Virol 66: 4784-4793.

132. Kuritzkes DR, Jacobson J, Powderly WG, Godofsky E, DeJesus E, et al. (2004) Antiretroviral activity of the anti-CD4 monoclonal antibody TNX-355 in patients infected with HIV type 1. J Infect Dis 189: 286-291.

133. Jacobson JM, Kuritzkes DR, Godofsky E, DeJesus E, Larson JA, et al. (2008) Safety, Pharmacokinetics, and Antiretroviral Activity of Multiple Doses of Ibalizumab (formerly TNX-355), an Anti-CD4 Monoclonal Antibody, in HIV-1 Infected Adults. Antimicrob Agents Chemother Published online ahead of print 17 November 2008

134. Pastore C, Picchio GR, Galimi F, Fish R, Hartley O, et al. (2003) Two mechanisms for human immunodeficiency virus type 1 inhibition by N-terminal modifications of RANTES. Antimicrob Agents Chemother 47: 509-517.

135. Mack M, Luckow B, Nelson PJ, Cihak J, Simmons G, et al. (1998) Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. J Exp Med 187: 1215-1224.

136. Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, et al. (1999) A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. Proc Natl Acad Sci U S A 96: 5698-5703.

137. Dorr P, Westby M, Dobbs S, Griffin P, Irvine B, et al. (2005) Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob Agents Chemother 49: 4721-4732.

138. Willey S, Peters PJ, Sullivan WM, Dorr P, Perros M, et al. (2005) Inhibition of CCR5-mediated infection by diverse R5 and R5X4 HIV and SIV isolates using novel small molecule inhibitors of CCR5: effects of viral diversity, target cell and receptor density. Antiviral Res 68: 96-108.

139. Strizki JM, Tremblay C, Xu S, Wojcik L, Wagner N, et al. (2005) Discovery and characterization of vicriviroc (SCH 417690), a CCR5 antagonist with potent

activity against human immunodeficiency virus type 1. Antimicrob Agents Chemother 49: 4911-4919.

140. Donzella GA, Schols D, Lin SW, Este JA, Nagashima KA, et al. (1998) AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nat Med 4: 72-77.

141. Este J, Telenti A (2007) HIV Entry Inhibitors. The Lancet in press.

142. Gulick RM, Su Z, Flexner C, Hughes MD, Skolnik PR, et al. (2007) Phase 2 Study of the Safety and Efficacy of Vicriviroc, a CCR5 Inhibitor, in HIV-1-Infected, Treatment-Experienced Patients: AIDS Clinical Trials Group 5211. J Infect Dis 196: 304-312.

143. J Lalezari JG, E DeJesus, H Lampiris, R Gulick, M Saag, C Ridgway, M McHale, E van der Ryst, and Howard Mayer (2007) Efficacy and Safety of Maraviroc plus Optimized Background Therapy in Viremic ART-experienced Patients Infected with CCR5-tropic HIV-1: 24-Week Results of a Phase 2b/3 Study in the US and Canada. 14th CROI.

144. M Nelson GF, I Konourina, A Lazzarin, N Clumeck, A Horban, M Tawadrous, J Sullivan, H Mayer, and Elna van der Ryst (2007) Efficacy and Safety of Maraviroc plus Optimized Background Therapy in Viremic, ART-experienced Patients Infected with CCR5-tropic HIV-1 in Europe, Australia, and North America: 24-Week Results. 14th CROI.

145. Kondru R, Zhang J, Ji C, Mirzadegan T, Rotstein D, et al. (2008) Molecular interactions of CCR5 with major classes of small-molecule anti-HIV CCR5 antagonists. Mol Pharmacol 73: 789-800.

146. Baba M, Takashima K, Miyake H, Kanzaki N, Teshima K, et al. (2005) TAK-652 inhibits CCR5-mediated human immunodeficiency virus type 1 infection in vitro and has favorable pharmacokinetics in humans. Antimicrob Agents Chemother 49: 4584-4591.

147. Takashima K, Miyake H, Kanzaki N, Tagawa Y, Wang X, et al. (2005) Highly potent inhibition of human immunodeficiency virus type 1 replication by TAK-220, an orally bioavailable small-molecule CCR5 antagonist. Antimicrob Agents Chemother 49: 3474-3482.

148. Strizki JM, Xu S, Wagner NE, Wojcik L, Liu J, et al. (2001) SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine

receptor CCR5, is a potent inhibitor of HIV-1 infection in vitro and in vivo. Proc Natl Acad Sci U S A 98: 12718-12723.

149. Schurmann D, Fatkenheuer G, Reynes J, Michelet C, Raffi F, et al. (2007) Antiviral activity, pharmacokinetics and safety of vicriviroc, an oral CCR5 antagonist, during 14-day monotherapy in HIV-infected adults. Aids 21: 1293-1299.

150. Landovitz RJ, Angel JB, Hoffmann C, Horst H, Opravil M, et al. (2008) Phase II study of vicriviroc versus efavirenz (both with zidovudine/lamivudine) in treatment-naive subjects with HIV-1 infection. J Infect Dis 198: 1113-1122.

151. Maeda K, Nakata H, Koh Y, Miyakawa T, Ogata H, et al. (2004) Spirodiketopiperazine-based CCR5 inhibitor which preserves CC-chemokine/CCR5 interactions and exerts potent activity against R5 human immunodeficiency virus type 1 in vitro. J Virol 78: 8654-8662.

152. Nakata H, Maeda K, Miyakawa T, Shibayama S, Matsuo M, et al. (2005) Potent anti-R5 human immunodeficiency virus type 1 effects of a CCR5 antagonist, AK602/ONO4128/GW873140, in a novel human peripheral blood mononuclear cell nonobese diabetic-SCID, interleukin-2 receptor gamma-chain-knocked-out AIDS mouse model. J Virol 79: 2087-2096.

153. Lalezari J, Thompson M, Kumar P, Piliero P, Davey R, et al. (2005) Antiviral activity and safety of 873140, a novel CCR5 antagonist, during short-term monotherapy in HIV-infected adults. Aids 19: 1443-1448.

154. Crabb C (2006) GlaxoSmithKline ends aplaviroc trials. Aids 20: 641.

155. Fatkenheuer G, Pozniak AL, Johnson MA, Plettenberg A, Staszewski S, et al. (2005) Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. Nat Med 11: 1170-1172.

156. Gulick RM, Lalezari J, Goodrich J, Clumeck N, DeJesus E, et al. (2008) Maraviroc for previously treated patients with R5 HIV-1 infection. N Engl J Med 359: 1429-1441.

157. Shin N SK, Wang KH, et al. (2007) INCB9471 is a non-competitive small molecule antagonist of CCR5. 47th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) 2007. Chicago, IL, USA

158. Solomon K BF, Shin N, et al. (2007) INCB9471 is a potent inhibitor of R5-HIV-1 infection in vitro. 47th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) Chicago, IL, USA.

159. Roschke V, Clarck, S., Branco, L., et al. (2004) Characterization of a panel of novel human monoclonal antibodies that specifically antagonize CCR5 and block HIV entry. 44th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). Washington, DC, USA.

160. Lalezari J, Yadavalli GK, Para M, Richmond G, Dejesus E, et al. (2008) Safety, pharmacokinetics, and antiviral activity of HGS004, a novel fully human IgG4 monoclonal antibody against CCR5, in HIV-1-infected patients. J Infect Dis 197: 721-727.

161. Olson WC, Rabut GE, Nagashima KA, Tran DN, Anselma DJ, et al. (1999) Differential inhibition of human immunodeficiency virus type 1 fusion, gp120 binding, and CC-chemokine activity by monoclonal antibodies to CCR5. J Virol 73: 4145-4155.

162. Jacobson JM, Saag MS, Thompson MA, Fischl MA, Liporace R, et al. (2008) Antiviral Activity of Single-Dose PRO 140, a CCR5 Monoclonal Antibody, in HIV-Infected Adults. J Infect Dis 198: 1345-1352.

163. Pugach P, Ketas TJ, Michael E, Moore JP (2008) Neutralizing antibody and anti-retroviral drug sensitivities of HIV-1 isolates resistant to small molecule CCR5 inhibitors. Virology 377: 401-407.

164. Kuhmann SE, Pugach P, Kunstman KJ, Taylor J, Stanfield RL, et al. (2004) Genetic and phenotypic analyses of human immunodeficiency virus type 1 escape from a small-molecule CCR5 inhibitor. J Virol 78: 2790-2807.

165. Marozsan AJ, Kuhmann SE, Morgan T, Herrera C, Rivera-Troche E, et al. (2005) Generation and properties of a human immunodeficiency virus type 1 isolate resistant to the small molecule CCR5 inhibitor, SCH-417690 (SCH-D). Virology 338: 182-199.

166. Murga JD, Franti M, Pevear DC, Maddon PJ, Olson WC (2006) Potent antiviral synergy between monoclonal antibody and small-molecule CCR5 inhibitors of human immunodeficiency virus type 1. Antimicrob Agents Chemother 50: 3289-3296.

167. Ji C, Zhang J, Dioszegi M, Chiu S, Rao E, et al. (2007) CCR5 small-molecule antagonists and monoclonal antibodies exert potent synergistic antiviral effects by cobinding to the receptor. Mol Pharmacol 72: 18-28.

168. Schols D, Esté JA, Henson G, De Clercq E (1997) Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. Antiviral Res 35: 147-156.

169. Doranz BJ, Grovit-Ferbas K, Sharron MP, Mao SH, Goetz MB, et al. (1997) A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. J Exp Med 186: 1395-1400.

170. Este JA, Cabrera C, De Clercq E, Struyf S, Van Damme J, et al. (1999) Activity of different bicyclam derivatives against human immunodeficiency virus depends on their interaction with the CXCR4 chemokine receptor. Mol Pharmacol 55: 67-73.

171. De Clercq E (2003) The bicyclam AMD3100 story. Nat Rev Drug Discov 2: 581-587.

172. Hendrix CW, Collier AC, Lederman MM, Schols D, Pollard RB, et al. (2004) Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. J Acquir Immune Defic Syndr 37: 1253-1262.

173. Moyle G, DeJesus E, Boffito M, Wong R, Coakley E, et al. CXCR4 Antagonism: Proof of activity wiht AMD11070.; 2007.

174. Hatse S, Princen K, De Clercq E, Rosenkilde MM, Schwartz TW, et al. (2005) AMD3465, a monomacrocyclic CXCR4 antagonist and potent HIV entry inhibitor. Biochem Pharmacol 70: 752-761.

175. Pettersson S, Perez-Nueno VI, Ros-Blanco L, Puig de La Bellacasa R, Rabal MO, et al. (2008) Discovery of novel non-cyclam polynitrogenated CXCR4 coreceptor inhibitors. ChemMedChem 3: 1549-1557.

176. Wong RS, Bodart V, Metz M, Labrecque J, Bridger G, et al. (2008) Comparison of the potential multiple binding modes of bicyclam, monocylam and non-cyclam small-molecule CXCR4 inhibitors. Mol Pharmacol.

177. Murakami T, Nakajima T, Koyanagi Y, Tachibana K, Fujii N, et al. (1997) A Small Molecule CXCR4 Inhibitor that Blocks T Cell Line-tropic HIV-1 Infection. J Exp Med 186: 1389-1393.

178. Arakaki R, Tamamura H, Premanathan M, Kanbara K, Ramanan S, et al. (1999) T134, a small-molecule CXCR4 inhibitor, has no cross-drug resistance with AMD3100, a CXCR4 antagonist with a different structure. J Virol 73: 1719-1723.

179. Doranz BJ, Filion LG, Diaz-Mitoma F, Sitar DS, Sahai J, et al. (2001) Safe use of the CXCR4 inhibitor ALX40-4C in humans. AIDS Res Hum Retroviruses 17: 475-486.

180. Daelemans D, Schols D, Witvrouw M, Pannecouque C, Hatse S, et al. (2000) A second target for the peptoid Tat/transactivation response element inhibitor CGP64222: inhibition of human immunodeficiency virus replication by blocking CXC-chemokine receptor 4-mediated virus entry. Mol Pharmacol 57: 116-124.

181. Ichiyama K, Yokoyama-Kumakura S, Tanaka Y, Tanaka R, Hirose K, et al. (2003) A duodenally absorbable CXC chemokine receptor 4 antagonist, KRH-1636, exhibits a potent and selective anti-HIV-1 activity. Proc Natl Acad Sci U S A 100: 4185-4190.

182. Choi WT, Tian S, Dong CZ, Kumar S, Liu D, et al. (2005) Unique ligand binding sites on CXCR4 probed by a chemical biology approach: implications for the design of selective human immunodeficiency virus type 1 inhibitors. J Virol 79: 15398-15404.

183. Princen K, Hatse S, Vermeire K, Aquaro S, De Clercq E, et al. (2004) Inhibition of human immunodeficiency virus replication by a dual CCR5/CXCR4 antagonist. J Virol 78: 12996-13006.

184. M Delmedico BB, N Cammack, D Davison, J Dwyer, L Frick, N Tvermoes, S Wring, H Zhang, and M Greenberg (2006) Next Generation HIV Peptide Fusion Inhibitor Candidates Achieve Potent, Durable Suppression of Virus Replication in vitro and Improved Pharmacokinetic Properties. 13th Conference on Retroviruses and Opportunistic Infections CROI. Denver.

185. Oishi S, Ito S, Nishikawa H, Watanabe K, Tanaka M, et al. (2008) Design of a novel HIV-1 fusion inhibitor that displays a minimal interface for binding affinity. J Med Chem 51: 388-391.

186. Dwyer JJ, Wilson KL, Davison DK, Freel SA, Seedorff JE, et al. (2007) Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus. Proc Natl Acad Sci U S A 104: 12772-12777.

187. He Y, Cheng J, Lu H, Li J, Hu J, et al. (2008) Potent HIV fusion inhibitors against Enfuvirtide-resistant HIV-1 strains. Proc Natl Acad Sci U S A.

188. Yu H, Tudor D, Alfsen A, Labrosse B, Clavel F, et al. (2008) Peptide P5 (residues 628-683), comprising the entire membrane proximal region of HIV-1 gp41 and

its calcium-binding site, is a potent inhibitor of HIV-1 infection. Retrovirology 5: 93.

189. Munch J, Standker L, Adermann K, Schulz A, Schindler M, et al. (2007) Discovery and optimization of a natural HIV-1 entry inhibitor targeting the gp41 fusion peptide. Cell 129: 263-275.

190. He Y, Xiao Y, Song H, Liang Q, Ju D, et al. (2008) Design and evaluation of sifuvirtide, a novel HIV-1 fusion inhibitor. J Biol Chem 283: 11126-11134.

191. Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. Nature 343: 425-430.

192. Fantoni M, Del Borgo C, Autore C (2003) Evaluation and management of metabolic and coagulative disorders in HIV-infected patients receiving highly active antiretroviral therapy. Aids 17 Suppl 1: S162-169.

193. Steinhart CR, Emons MF (2004) Risks of cardiovascular disease in patients receiving antiretroviral therapy for HIV infection: implications for treatment. AIDS Read 14: 86-90, 93-85.

194. Vittecoq D, Escaut L, Chironi G, Teicher E, Monsuez JJ, et al. (2003) Coronary heart disease in HIV-infected patients in the highly active antiretroviral treatment era. Aids 17 Suppl 1: S70-76.

195. Penzak SR, Chuck SK, Stajich GV (2000) Safety and efficacy of HMG-CoA reductase inhibitors for treatment of hyperlipidemia in patients with HIV infection. Pharmacotherapy 20: 1066-1071.

196. Maziere JC, Landureau JC, Giral P, Auclair M, Fall L, et al. (1994) Lovastatin inhibits HIV-1 expression in H9 human T lymphocytes cultured in cholesterol-poor medium. Biomed Pharmacother 48: 63-67.

197. Koch G, Benz C, Schmidt G, Olenik C, Aktories K (1997) Role of Rho protein in lovastatin-induced breakdown of actin cytoskeleton. J Pharmacol Exp Ther 283: 901-909.

198. Giguere JF, Tremblay MJ (2004) Statin compounds reduce human immunodeficiency virus type 1 replication by preventing the interaction between virion-associated host intercellular adhesion molecule 1 and its natural cell surface ligand LFA-1. J Virol 78: 12062-12065.

199. Palinski W (2000) Immunomodulation: a new role for statins? Nat Med 6: 1311-1312.

200. Kwak B, Mulhaupt F, Myit S, Mach F (2000) Statins as a newly recognized type of immunomodulator. Nat Med 6: 1399-1402.

201. Diomede L, Albani D, Sottocorno M, Donati MB, Bianchi M, et al. (2001) In vivo anti-inflammatory effect of statins is mediated by nonsterol mevalonate products. Arterioscler Thromb Vasc Biol 21: 1327-1332.

202. Bruegel M, Teupser D, Haffner I, Mueller M, Thiery J (2006) Statins reduce macrophage inflammatory protein-1alpha expression in human activated monocytes. Clin Exp Pharmacol Physiol 33: 1144-1149.

203. Wagner AH, Gebauer M, Guldenzoph B, Hecker M (2002) 3-hydroxy-3-methylglutaryl coenzyme A reductase-independent inhibition of CD40 expression by atorvastatin in human endothelial cells. Arterioscler Thromb Vasc Biol 22: 1784-1789.

204. Xu J, Lecanu L, Han Z, Yao Z, Greeson J, et al. (2003) Inhibition of adrenal cortical steroid formation by procaine is mediated by reduction of the cAMP-induced 3-hydroxy-3-methylglutaryl-coenzyme A reductase messenger ribonucleic acid levels. J Pharmacol Exp Ther 307: 1148-1157.

205. Westby M, Lewis M, Whitcomb J, Youle M, Pozniak AL, et al. (2006) Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. J Virol 80: 4909-4920.

206. Westby M, Smith-Burchnell C, Mori J, Lewis M, Mosley M, et al. (2007) Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. J Virol 81: 2359-2371.

207. Trkola A, Kuhmann SE, Strizki JM, Maxwell E, Ketas T, et al. (2002) HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. Proc Natl Acad Sci U S A 99: 395-400.

208. Baba M, Miyake H, Wang X, Okamoto M, Takashima K (2007) Isolation and characterization of human immunodeficiency virus type 1 resistant to the small-molecule CCR5 antagonist TAK-652. Antimicrob Agents Chemother 51: 707-715.

209. Pugach P, Marozsan A, Ketas T, Landes E, Moore J, et al. (2008) HIV-1 clones resistant to a small molecule CCR5 inhibitor use the inhibitor-bound form of CCR5 for entry. Virology In Press, Corrected Proof.

210. Ogert RA, Wojcik L, Buontempo C, Ba L, Buontempo P, et al. (2008) Mapping resistance to the CCR5 co-receptor antagonist vicriviroc using heterologous chimeric HIV-1 envelope genes reveals key determinants in the C2-V5 domain of gp120. Virology 373: 387-399.

211. Fatkenheuer G, Nelson M, Lazzarin A, Konourina I, Hoepelman AI, et al. (2008) Subgroup analyses of maraviroc in previously treated R5 HIV-1 infection. N Engl J Med 359: 1442-1455.

212. Heera J SM, Ive P, et al. (2008) Virological correlates associated with treatment failure at week 48 in the phase 3 study of maraviroc in treatment-naïve patients. 15th Conference on Retroviurses and Opportunistic Infections, Boston, MA Abstract 40 LB.

213. Tsibris AM, Sagar M, Gulick RM, Su Z, Hughes M, et al. (2008) In vivo emergence of vicriviroc resistance in a human immunodeficiency virus type 1 subtype C-infected subject. J Virol 82: 8210-8214.

214. Skrabal K, Low AJ, Dong W, Sing T, Cheung PK, et al. (2007) Determining human immunodeficiency virus coreceptor use in a clinical setting: degree of correlation between two phenotypic assays and a bioinformatic model. J Clin Microbiol 45: 279-284.

215. Whitcomb JM, Huang W, Fransen S, Limoli K, Toma J, et al. (2007) Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. Antimicrob Agents Chemother 51: 566-575.

216. Trouplin V, Salvatori F, Cappello F, Obry V, Brelot A, et al. (2001) Determination of coreceptor usage of human immunodeficiency virus type 1 from patient plasma samples by using a recombinant phenotypic assay. J Virol 75: 251-259.

217. Braun P, Wiesmann F (2007) Phenotypic assays for the determination of coreceptor tropism in HIV-1 infected individuals. Eur J Med Res 12: 463-472.

218. Weber J, Piontkivska H, Quinones-Mateu ME (2006) HIV type 1 tropism and inhibitors of viral entry: clinical implications. AIDS Rev 8: 60-77.

219. Coakley E, Petropoulos CJ, Whitcomb JM (2005) Assessing chemokine co-receptor usage in HIV. Curr Opin Infect Dis 18: 9-15.

220. Reeves J HD, Wilkin T, et al. An enhanced version of the Trofile HIV co-receptor tropism assay predicts emergence of CXCR4 use in ACTG5211 vicriviroc trial samples. 15th Conference on Retroviruses and Opportunistic Infections 2008; Feb 3-6, 2008; Boston, MA.

221. Jensen MA, Li FS, van 't Wout AB, Nickle DC, Shriner D, et al. (2003) Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. J Virol 77: 13376-13388.

222. Delobel P, Nugeyre MT, Cazabat M, Pasquier C, Marchou B, et al. (2007) Population-based sequencing of the V3 region of env for predicting the coreceptor usage of human immunodeficiency virus type 1 quasispecies. J Clin Microbiol 45: 1572-1580.

223. Jensen MA, Coetzer M, van 't Wout AB, Morris L, Mullins JI (2006) A reliable phenotype predictor for human immunodeficiency virus type 1 subtype C based on envelope V3 sequences. J Virol 80: 4698-4704.

224. Resch W, Hoffman N, Swanstrom R (2001) Improved success of phenotype prediction of the human immunodeficiency virus type 1 from envelope variable loop 3 sequence using neural networks. Virology 288: 51-62.

225. Blanco J, Clotet-Codina I, Bosch B, Armand-Ugon M, Clotet B, et al. (2005) Multiparametric assay to screen and dissect the mode of action of anti-human immunodeficiency virus envelope drugs. Antimicrob Agents Chemother 49: 3926-3929.

226. DeMarco SJ, Henze H, Lederer A, Moehle K, Mukherjee R, et al. (2006) Discovery of novel, highly potent and selective beta-hairpin mimetic CXCR4 inhibitors with excellent anti-HIV activity and pharmacokinetic profiles. Bioorg Med Chem 14: 8396-8404.

227. Armand-Ugon M, Gutierrez A, Clotet B, Este JA (2003) HIV-1 resistance to the gp41-dependent fusion inhibitor C-34. Antiviral Res 59: 137-142.

228. Cabana M, Clotet B, Martinez MA (1999) Emergence and genetic evolution of HIV-1 variants with mutations conferring resistance to multiple reverse transcriptase and protease inhibitors. J Med Virol 59: 480-490.

229. de Vreese K, Kofler-Mongold V, Leutgeb C, Weber V, Vermeire K, et al. (1996) The molecular target of bicyclams, potent inhibitors of human immunodeficiency virus replication. J Virol 70: 689-696.

230. Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, et al. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J Virol Methods 20: 309-321.

231. NIAID NaCI (1997) NIAID and NIH Virology Manual for HIV Laboratories.

232. Grivel JC, Margolis LB (1999) CCR5- and CXCR4-tropic HIV-1 are equally cytopathic for their T-cell targets in human lymphoid tissue. Nat Med 5: 344-346.

233. Armand-Ugón M, Clotet-Codina I, Tintori C, Manetti F, Clotet B, et al. (2005) The anti-HIV activity of ADS-J1 targets the HIV-1 gp120. Virology 343: 141-149.

234. Armand-Ugon M, Gutierrez A, Clotet B, Este JA (2003) HIV-1 resistance to the gp41-dependent fusion inhibitor C-34. Antiviral Res 59: 137-142.

235. Este JA, Schols D, De Vreese K, Van Laethem K, Vandamme AM, et al. (1997) Development of resistance of human immunodeficiency virus type 1 to dextran sulfate associated with the emergence of specific mutations in the envelope gp120 glycoprotein. Mol Pharmacol 52: 98-104.

236. Este JA, Cabrera C, Blanco J, Gutierrez A, Bridger G, et al. (1999) Shift of clinical human immunodeficiency virus type 1 isolates from X4 to R5 and prevention of emergence of the syncytium-inducing phenotype by blockade of CXCR4. J Virol 73: 5577-5585.

237. Pastore C, Ramos A, Mosier DE (2004) Intrinsic obstacles to human immunodeficiency virus type 1 coreceptor switching. J Virol 78: 7565-7574.

238. Yi Y, Shaheen F, Collman RG (2005) Preferential use of CXCR4 by R5X4 human immunodeficiency virus type 1 isolates for infection of primary lymphocytes. J Virol 79: 1480-1486.

239. Schols D, Este JA, Cabrera C, De Clercq E (1998) T-cell-line-tropic human immunodeficiency virus type 1 that is made resistant to stromal cell-derived factor 1alpha contains mutations in the envelope gp120 but does not show a switch in coreceptor use. J Virol 72: 4032-4037.

240. Kanbara K, Sato S, Tanuma J, Tamamura H, Gotoh K, et al. (2001) Biological and genetic characterization of a human immunodeficiency virus strain resistant to CXCR4 antagonist T134. AIDS Res Hum Retroviruses 17: 615-622.

241. Armand-Ugon M, Quinones-Mateu ME, Gutierez A, Barretina J, Blanco J, et al. (2003) Reduced fitness of HIV-1 resistant to CXCR4 antagonists. Antivir Ther 8: 1-8.

242. Moncunill G, Armand-Ugon M, Clotet B, Este JA (2008) HIV coreceptor switch induced by antagonism to CCR5. AIDS 22: 23-31.

243. Sinensky M, Beck LA, Leonard S, Evans R (1990) Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis. J Biol Chem 265: 19937-19941.

244. Zhang FL, Casey PJ (1996) Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem 65: 241-269.

245. Maltese WA (1990) Posttranslational modification of proteins by isoprenoids in mammalian cells. Faseb J 4: 3319-3328.

246. Etienne-Manneville S, Hall A (2002) Rho GTPases in cell biology. Nature 420: 629-635.

247. Quesney-Huneeus V, Wiley MH, Siperstein MD (1979) Essential role for mevalonate synthesis in DNA replication. Proc Natl Acad Sci U S A 76: 5056-5060.

248. Sklar PA, Masur H, Grubb JR, Voell J, Witek J, et al. (2005) Pravastatin does not have a consistent antiviral effect in chronically HIV-infected individuals on antiretroviral therapy. Aids 19: 1109-1111.

249. Waters L, Stebbing J, Jones R, Mandalia S, Bower M, et al. (2005) The effect of statins on HIV rebound and blips. J Acquir Immune Defic Syndr 39: 637-638.

250. Manfredi R, Calza L, Chiodo F (2006) Long-term statin use does not act on the temporal trend of CD4 cell count in patients on virologically effective HAART. Aids 20: 455-457.

251. Negredo E, Clotet B, Puig J, Perez-Alvarez N, Ruiz L, et al. (2006) The effect of atorvastatin treatment on HIV-1-infected patients interrupting antiretroviral therapy. Aids 20: 619-621.

252. Garcia MJ, Reinoso RF, Sanchez Navarro A, Prous JR (2003) Clinical pharmacokinetics of statins. Methods Find Exp Clin Pharmacol 25: 457-481.

253. Lennernas H, Fager G (1997) Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences. Clin Pharmacokinet 32: 403-425.

254. Moghadasian MH (2002) A safety look at currently available statins. Expert Opin Drug Saf 1: 269-274.

255. Sinzinger H, Wolfram R, Peskar BA (2002) Muscular side effects of statins. J Cardiovasc Pharmacol 40: 163-171.

256. Backes JM, Howard PA (2003) Association of HMG-CoA reductase inhibitors with neuropathy. Ann Pharmacother 37: 274-278.

257. Golomb BA, Kane T, Dimsdale JE (2004) Severe irritability associated with statin cholesterol-lowering drugs. Qjm 97: 229-235.

258. Wagstaff LR, Mitton MW, Arvik BM, Doraiswamy PM (2003) Statin-associated memory loss: analysis of 60 case reports and review of the literature. Pharmacotherapy 23: 871-880.

259. Edison RJ, Muenke M (2004) Central nervous system and limb anomalies in case reports of first-trimester statin exposure. N Engl J Med 350: 1579-1582.

260. Corsini A (2003) The safety of HMG-CoA reductase inhibitors in special populations at high cardiovascular risk. Cardiovasc Drugs Ther 17: 265-285.

261. Jensen MA, van 't Wout AB (2003) Predicting HIV-1 coreceptor usage with sequence analysis. AIDS Rev 5: 104-112.

262. Fernandez G, Llano A, Esgleas M, Clotet B, Este JA, et al. (2006) Purifying selection of CCR5-tropic human immunodeficiency virus type 1 variants in AIDS subjects that have developed syncytium-inducing, CXCR4-tropic viruses. J Gen Virol 87: 1285-1294.

263. Reeves JD, Gallo SA, Ahmad N, Miamidian JL, Harvey PE, et al. (2002) Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. Proc Natl Acad Sci U S A 99: 16249-16254.

264. Hoffman NG, Seillier-Moiseiwitsch F, Ahn J, Walker JM, Swanstrom R (2002) Variability in the human immunodeficiency virus type 1 gp120 Env protein linked to phenotype-associated changes in the V3 loop. J Virol 76: 3852-3864.

265. Voulgaropoulou F, Tan B, Soares M, Hahn B, Ratner L (1999) Distinct human immunodeficiency virus strains in the bone marrow are associated with the development of thrombocytopenia. J Virol 73: 3497-3504.

266. Troyer RM, Collins KR, Abraha A, Fraundorf E, Moore DM, et al. (2005) Changes in human immunodeficiency virus type 1 fitness and genetic diversity during disease progression. J Virol 79: 9006-9018.

267. Shankarappa R, Margolick JB, Gange SJ, Rodrigo AG, Upchurch D, et al. (1999) Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J Virol 73: 10489-10502.

268. Sophie Holuigue MS, T Ketas, and J Moore (2007) Co-receptor Switched and CCR5 Inhibitor-resistant HIV-1 Variants Are Generated in Response to Different Selection Pressures in vitro. 14th CROI.

269. van Rij RP, Hazenberg MD, van Benthem BH, Otto SA, Prins M, et al. (2003) Early viral load and CD4+ T cell count, but not percentage of CCR5+ or CXCR4+ CD4+ T cells, are associated with R5-to-X4 HIV type 1 virus evolution. AIDS Res Hum Retroviruses 19: 389-398.

270. Harrison JE, Lynch JB, Sierra LJ, Blackburn LA, Ray N, et al. (2008) Baseline resistance of primary HIV-1 strains to the CXCR4 inhibitor AMD3100. J Virol.

271. Moyle GJ, Wildfire A, Mandalia S, Mayer H, Goodrich J, et al. (2005) Epidemiology and predictive factors for chemokine receptor use in HIV-1 infection. Journal of Infectious Diseases 191: 866-872.

272. Brumme ZL, Goodrich J, Mayer HB, Brumme CJ, Henrick BM, et al. (2005) Molecular and clinical epidemiology of CXCR4-using HIV-1 in a large population of antiretroviral-naive individuals. Journal of Infectious Diseases 192: 466-474.

273. Melby T, DeSpirito M, DeMasi R, Heilek-Snyder G, Greenberg ML, et al. (2006) HIV-1 coreceptor use in triple-class treatment experienced patients: Baseline prevalence, correlates, and relationship to enfuvirtide response. Journal of Infectious Diseases 194: 238-246.

274. Weber J, Piontkivska H, Quiñones-Mateu ME (2006) HIV Type 1 Tropism and Inhibitors of Viral Entry: Clinical Implications. AIDS Reviews 8: 60-77.

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