HIV tropism and its implications for antiretroviral therapy

Tropismul HIV și implicațiile acestuia în terapia antiretrovirală

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Abstract

Viral tropism defines the feature of HIV isolates to use a certain CCR5, CXCR4 coreceptor in order to enter CD4 positive, receptor cells. The dynamics of the HIV tropism plays a major role in the pathogenesis of the HIV infection and influences the progression of the disease. HIV tropism correlates with the epidemiology of HIV-1 subtypes and can be modified by the antiretroviral complex therapy. Coreceptor antagonists CCR5 (CXCR4) represent a new generation of molecules used in the therapy of HIV infection, in multi-experienced patients as well as in naïve individuals. Maraviroc is the only drug from the class of coreceptor antagonists approved for the treatment of CCR5 tropic HIV-1 infected patients. In order to determine the susceptibility of an HIV-1 population to CCR5 antagonists, viral tropism testing is indispensable. Several phenotype and genotype tests were developed to identify the tropism of the HIV isolates. The clinically approved method is Trofile, certified according to the specifications of the Clinical Laboratory Improvement Amendments (CLIA).

Keywords: CCR5, CXCR4 coreceptors, HIV-1 tropism, coreceptor antagonists

Rezumat


Cuvinte cheie: Coreceptori CCR5, CXCR4, tropismul HIV-1, antagoniști de coreceptori

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The development of antiretroviral therapy is one of the most remarkable achievements in the history of medicine. Starting in 1987 with the approval of Zidovudine, great progress has been made in this area, including the adoption of HAART (highly active antiretroviral therapy) concept in 1996, which transformed HIV (Human Immunodeficiency Virus) infection into a chronic, controllable infection. Currently, the adherent patient has a large spectrum of antiretrovirals at his or her disposal (1). Unfortunately, the adverse reactions have not waited long to appear. The toxicity derived from long term use of certain drugs, resistance mutations which appeared to a certain class of drugs, cross resistance with other classes of antiretrovirals limit successful therapeutic support of these molecules. Research for new molecules continued, and thus new classes of antiretrovirals were discovered, which have as target different stages of the viral replication cycle: fusion inhibitors, integration inhibitors, maturation inhibitors, coreceptor antagonists. HIV penetration into target cells represents an attractive stage for the development of new therapeutic agents. HIV adsorption and entry into the cells expressing CD4 receptors implies the development of several very well ordered events, which begins with the sequential interaction between viral envelope glycoproteins (gp120, gp41) and specific receptors on the surface of the target cell: CD4 and two coreceptors: CCR5 (cysteine-cysteine-receptor 5), CXCR4 (cysteine-X-cysteine-receptor 4) (2). Maraviroc is the only antagonist of CCR5 coreceptor approved in the therapy of HIV-positive patients. It is the only orally administered HIV entry inhibitor. Maraviroc inhibits replication of HIV-R5 tropic variants by allosteric mechanism after binding with CCR5 chemokine coreceptor (3, 4). Vicriviroc is in the third phase of clinical study, and is also an inhibitor of R5 viruses, similar to Maraviroc. It interacts with the CCR5 transmembranal domain, inhibiting gp120 attachment by allosteric mechanism. The third CCR5 inhibitor, Aplaviroc, was withdrawn because of the high risk of hepatotoxicity (4).

**HIV Tropism**

HIV tropism was defined starting with 1980, when the existence of different virus variants was established. Initially, “tropism” referred to the cytopathic effect of HIV isolates on circulating mononuclear cells. The viral stems which produced syncytia (multinucleated giant T-cells) in the mononuclear cells were defined as syncytium inducing (SI), in contrast with the non-syncytium inducing (NSI) ones (2). Based on the replication kinetics within the peripheral mononuclear blood cells (PMBC), HIV isolates were classified as slow-low and rapid-high inducing stems. A new classification was suggested recently, considering the ability of the virus to replicate in two cellular lines: macrophages, derivatives of monocytes or CD4 lymphocytes: M-tropic, respectively, T-tropic viruses. In 1996, it was discovered that in the stage of adsorption and penetration into target cell, HIV resorts to coreceptors (1, 2, 4, 5). The CCR5 and CXCR4 coreceptors belong to the family of chemokine receptors. Chemokines are polypeptides acting as signaling molecules by connecting to the chemokine receptors. They are part of the cytokine family, and they initiate the process of chemotaxis. Chemokine receptors have seven transmembrane domains and belong to the G protein-coupled receptor class. They present an α-helix structure composed of four transmembrane domains - three extracellular loops and one N-terminal domain. The activated chemokine receptors, with a degree of specificity and affinities, develop a complex network of interactions which play a fundamental role in human pathology (4-6). Chemokine binding causes a conformational change in the receptor that triggers a cascade of intracellular responses and processes. It is known that in the early stages of HIV-1 infection, viral isolates...
tend to use CCR5 and in more advanced stages they use coreceptor CXCR4 (7). Isolates classified as NS1 or M-tropic use coreceptor CCR5 in order to penetrate cells. Isolates which use CXCR4 are SI and T-tropic. This finding allowed a new definition of viral tropism, based on the type of coreceptor used, which splits HIV isolates into three categories: CCR5 – tropic (R5), CXCR4 – tropic (X4), dual-mix tropic R5-X4 (2,4,5). Besides the crucial step in HIV entry, chemokines and chemokine receptors play an important role in the distribution of effector cells to sites of microbial infection where they may contribute to microbial control and elimination; they play a major role in autoimmune diseases also. CCR5 receptors can be activated by many chemokines, for example MIP-1-alfa, MIP-1-beta (macrophage inflammatory protein-1-alfa, 1-beta) and RANTES (regulated on activation normal T-cell expressed and secreted). CXCR4 receptors are activated solely by stromal cell-derived factor-1. Chemokine receptors seem to be playing an important role in the hepatitis C virus (HCV) infection. Increased density of this chemokine receptor in the surface of T-cells stimulates the intensity of the inflammatory response and the accumulation of T-cells in the hepatic tissue (2). Some authors have demonstrated the correlation between low CCR5 levels and the more favorable evolution of HCV infection through a higher viral clearance and reduction of inflammation. Shutting off the chemokine receptor involved in the immune response can be associated with increasing the risk of developing certain infections or generating certain malignancies (8). The lack of support of these suppositions derives from the fact that individuals with CCR5 genetic deficit are healthy, although some studies proved severe infections with West Nile virus in these individuals (6). Deletion of CXCR4 results in embryonic lethality owing to hematopoietic, cerebellar, gastrointestinal and cardiac defects (6). Total absence of CCR5, in certain homozygous subjects, for the delta CCR5 defective allele is protective against HIV infection. Heterozygous individuals who carry delta 32 mutation in one copy of the CCR5 gene show a reduced expression of CCR5 on the surface of cells, can be infected with HIV, but prove a delay in TCD4 cell depletion and the progression of HIV infection is slowed down (2, 5, 9). HIV virus adsorption and entry in cells implies several very well ordered events, which begin by involving CD4 molecule, followed by coreceptors and fusion. Gp120 attaches to CD4 receptors, inducing conformational changes in gp120 and exposing a binding situs to coreceptors. CD4-gp120 complex binds to coreceptors through the V3 hypervariable domain of gp120, also involving in this interaction regions such as V1/V2 and C4. The amino acid sequences of V3 seem to be major determinants of coreceptor usage. The process map suggests that, for R5 viruses, the N-terminal domain and the second extracellular domain (ECL2) are essential for recognizing coreceptors and functional activation. For X4 variants only ECL2 domain is important (1, 2, 5).

Epidemiology

There are limited data regarding the correlation between the HIV subtype and the epidemiology of coreceptor tropism. Recent studies suggest that viral subtype D can be associated with the more frequent use of CXCR4 coreceptor. Clinical implications are not very well defined, but subtype D infected patients can manifest a higher risk of disease progression (7, 10). Also, a tight correlation was proven between the number of CD4 cells and the virus infection using CXCR4. 10% of the individuals infected with such a viral subtype have values of T CD4 of 500 cells/mm$^3$, and approximately 50% display CD4 values below 200 cells/mm$^3$. The prevalence of this virus is higher in multireexperienced patients. Other factors associated with increased incidence of CXCR4 virus infection include: low level of
natural killer cells, viral subtype D, heterozygous individuals for CCR5d32, or those expressing the stromal cell-derived factor-1 (7, 10).

**Dynamics of HIV tropism**

The pattern and extension of coreceptors expression on the surface of human cells influences the dynamics of viral tropism. In the evolution of HIV infection, R5 variants are generally responsible for establishing the infection and are transmitted more efficiently than X4 variants. In the advanced stages of infection X4 variants are more efficient; they are associated with progression towards AIDS and rapid depletion of CD4 lymphocytes (11). Homozygous individuals for the 32-base pair deletion of the CCR5 gene seem to be protected from HIV infection, even if they are exposed several times. It is indirectly suggested that R5 stems are prevalent in the early stages of the infection and in viral transmission. The question arises: why R5 isolates prevail in the early stages of the infection, even if the ways of transmission are different: sexual, vertical or parenteral. R5 HIV variants have the advantage of expression of CCR5 coreceptor in important quantities at the level of genital mucous membranes. Although X4 viruses seem to be more efficiently transmitted by parenteral exposure, within the same population, R5 variants prevail in early stages of infection (2, 11, 12). HIV may infect CCR5-expressing dendritic cells (DC), macrophages and T-cells. DC can express CD4, CCR5, DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3 Grabbing Non-integrin) receptors, and other C-types lectin receptors which facilitate HIV or SIV capture and infection (2). DC can capture HIV through C-type lectin receptors and can lead to productive infection though a CCR5-dependent mechanism (cis infection). DC also captures and transfers HIV by binding DC-SIGN/CD209 to carbohydrates on gp120, without becoming infected (trans-transmission model). DC are responsible for the trafficking of HIV particles to the lymph nodes. When HIV particles reach the lymphoid tissue they induce the activation of TCD4-cells which acquire a memory subset, which expresses higher CCR5 than CXCR4 levels. These facts could explain the preferential selection of R5 viruses following initial exposure. X4 viruses have the ability to infect thymocytes, the precursor cells of mature CD4T-lymphocytes, affecting thymopoiesis. This fact may explain the rapid CD4T-cell depletion, and progression to AIDS, generally seen in patients with X4 viruses (4, 7-9).

**The impact of antiretroviral therapy on HIV tropism**

HAART therapy has dramatically changed the natural evolution of the HIV infection, the expectation of life of HIV-infected patients now being almost the same as that of non-infected individuals. The development of new drugs against HIV has resulted in combination therapy that can inhibit various steps of viral replication. Maraviroc has show activity both in vitro and in vivo against R5 HIV strains. In two large trials: MOTIVATE1 (conducted in North America) and MOTIVATE2 (conducted in Europe) administration of maraviroc along with optimized background therapy to treatment-experienced patients harbouring R5 viruses at baseline resulted in significant decrease in viral load and significant increase in CD4-cell counts (1,8,9). Both studies enrolled patients aged 16 years or older with only R5 HIV-1 detected at screening using the original Trofile assay (Monogram Biosciences, South San Francisco, CA), plasma HIV-1 RNA > 5000 copies per milliliter and experienced with or resistant to at least 3 antiretroviral drug classes. Maraviroc, as compared with placebo, resulted in significantly greater suppression of HIV-1 viral load and greater increases in CD4-cell counts at 48 weeks in previously treated patients with R5 HIV-1 who were receiving an optimized background therapy (1, 8, 9). The MERIT trial eval-
uated the safety and efficacy of maraviroc used twice daily as compared with that of efavirenz once daily, both with zidovudine and lamivudine, as initial therapy in drug-naïve HIV-infected patients. At 48 weeks, in an intent-to-treat evaluation, the trial failed to demonstrate non-inferiority of maraviroc arms relative to efavirenz, using the attainment of <50 copies/ml HIV-RNA viral load as secondary endpoint. When the subjects were reassessed using the new enhanced tropism assay, 15% of patients who had R5 virus at screening by the old assay were reclassified as dual/mixed tropic. When those patients were excluded from the efficacy analysis, similar responses were seen on comparing maraviroc and efavirenz groups (11, 12, 14). Currently, there is a preoccupation regarding a potential influence of ARV therapy on the selection of HIV X4 variants (12). The impact of antiretroviral therapy use on the dynamics of viral tropism is still poorly understood. In patients receiving CCR5 antagonists, virus strains that no longer respond to the CCR5 antagonist may emerge by 2 mechanisms. The first mechanism is the emergence of viral variants that can use CXCR4. These viruses may be dual tropic (R5/X4) strains or they use CXCR4 exclusively. Studies have shown that even a small minority of X4 virus can be associated with failure of CCR5 antagonist therapy. The second mechanism consists of the emergence of drug-resistant viral strains. The higher frequency of X4 variants in antiretroviral-experienced patients was associated with the lower CD4 count. The preferential sites of replication for X4 viruses are the thymocytes and the naïve T CD4 lymphocytes, where the production of residual virus explains the emergence of X4 variants in the cellular reservoirs in patients on HAART. Another reason explaining the switch from R5 to X4 under HAART is the peripheral expansion of CD45RA+ long-lived T-cells which deposit X4 virus. The spread of X4 variants and infection of thymocytes and CD4 naïve cells compromise the immune reconstruction under HAART. X4 viruses emerge as a consequence of progressive immune damage (14). In spite of a well defined correlation between the progression of HIV infection and the use of coreceptors, viral tropism determination is not currently a routine in the follow-up of HIV-infected patients. Plasma viral load and CD4 cells count continue to be the main parameters for prognosis and therapeutic decision, but the development of CCR5 antagonists has modified this paradigm. The inclusion of antagonists in the therapeutic arsenal requires knowing the HIV coreceptor used by each patient. Tropism testing must be performed before a CCR5 antagonist can be included in a patients’ antiretroviral therapy. Maraviroc is the only approved CCR5 antagonist for treatment-experienced adult patients who have only CCR5-tropic HIV-1 detectable and have evidence of viral replication and resistance to multiple antiretroviral agents. Because of their action mechanism, the benefit of coreceptor antagonists is limited to a subset of HIV-infected patients - those with only R5 virus variants. For the moment, the effect of the inhibition of these coreceptors is insufficiently specified (13-15).

**Methods to determine HIV tropism**

Coreceptor HIV-1 tropism testing can be performed by two different methods: phenotypic and genotypic methods.

**Phenotypic Methods**

The phenotypic methods can be classical or recombinant techniques. A traditional method of determining the HIV-1 tropism originated in tests assessing viral phenotypes in cell cultures, since the 1980s. MT-2 assay was used initially to determine the phenotype of syncytium-inducing virus subtypes (SI), known today as X4 or R5X4 dual phenotypes tropics, and those non-syncytium-inducing (NIS-viruses, CCR5-tropics). This test is based on the existence of cell surface receptor CXCR4. Viral replication in these cells was highlighted by the
appearance of giant T-cells (syncytia), which demonstrate the existence of X4 coreceptors. A major disadvantage of the method is the need of using viral concentrates obtained from stimulated patient PMBC. PMBC derived from HIV-infected patients were incubated with cells from HIV-negative donors, stimulated with CD3/CD28 antibodies, phytohemagglutinin or in the presence of interleukin 2 (16).

Trofile (Monogram Biosciences, South San Francisco, California, USA)

This method is clinically validated by the Clinical Laboratory Improvements Act (CLIA) standards for the determination of HIV tropism in patients following treatment with CCR5 antagonists (7, 17). The principle at the basis of Trofile is similar to that of recombinant phenotype assays used for evaluating antiretroviral resistance. The genes of HIV envelope isolated from the patient’s plasma are amplified by a polymerase chain reaction (PCR) and then inserted into envelope expression vectors. In another stage, the envelope expression vectors together with the HIV reporting vector which contains a gene for the luciferase indicator are introduced in cellular cultures. Pseudoviruses are produced in these cultures, expressing both a luciferase indicator and HIV-1 envelope glycoproteins derived from viral strains isolated from the patient’s plasma. Pseudoviruses, capable of a single round of replication, are used to infect the target T-cellular lines which express CD4 and CCR5 or CXCR4. Viral replication determines luciferase production which can be quantified by measuring the relative light units (RLU) emitted by the infected cells. Luciferase production in the CCR5 or CXCR4 cells defines the tropism of the HIV isolates. Entry inhibitors, CCR5 or CXCR4 antagonists, can be introduced in the culture environment in order to confirm the tropism type. A viral population composed of R5 virus will determine a positive result only in the target T-cellular lines which express CCR5, while in the populations composed of X4 virus, a positive result is obtained only in the target T-cellular lines which express CXCR4. The R5/X4 dual-mixed viral population will produce signal in both cellular lines. The method is not capable to make the difference between the two populations. Tropism evaluation tests and other biological methods have an inferior detection limit below which minority species remain unidentified. The tests require a minimum viral load of 1000 copies/ml in order to ensure a valid result. The tropism evaluation tests cannot make the difference between HIV-1 isolates with pure R5 or X4 tropism and with mixed tropism and they cannot evaluate the expansion of X4 virus. In order to determine if the patient’s virus has dual or mixed tropism they are individually tested by clonal analysis of a large number of viral particles, an extremely laborious and expensive process. The methods currently used to determine coreceptor tropism can detect minority variants only if they are present in concentrations above a certain threshold value. Consequently, it may be possible for the patients identified as having only R5 virus to actually be bearers of an undetectable population of viruses using CXCR4 (7, 17, 18). Monogram Biosciences recently developed (August 2008) a more advanced version of the Trofile technique, capable to detect X4 variants even in minority proportions, over 0,1 – 0,3% of the viral population (7).

HIV-1 Phenoscript Env™ Method (VIRalliance, Paris, France)

This test determines both viral tropism and sensitivity to inhibitors of virus fusion. It uses recombinant viral particles produced by homologous recombination of the following sequences: a linear vector, pNL4-3 lacking env gene and amplified env region, derived from plasma taken from patients. Recombinant viral particles are produced using T-HEK-293 cells.

Determination of viral tropism and resistance is performed by using a 2180 bp env sequence for tropism or resistance testing, and a 900 bp sequence of the V1-V3 region for determining viral tropism only. The obtained amplicon is inserted in both cases into the pNL4-3 plasmid lacking the env gene (gp 120
and the ectodomain of gp4, positions 6480 to 8263). The obtained \textit{env} recombinant viruses are replication-competent and are obtained by homologous recombination in HEK293-T-cells. The recombinant viruses are transfected in U373MG-CD4 indicator cells that express either receptor CCR5/CD4 or receptor CXCR4/CD4. The method allows assessment of infectivity in a single cycle, completed by a colorimetric assay based on beta-galactosidase expression induced by HIV-1Tat. The method has the advantage of parallel determination of HIV tropism and resistance to viral fusion inhibitors. This method can evaluate HIV tropism in non-subtype B group (16).

\textbf{Xtrack} and \textbf{PhenX-R (InPheno AG, Basel, Switzerland)}

This method combines a hybridization based genosorting (XtrackC) with a replicative determination of viral phenotype (PhenX-R). The first stage allows a quick test to identify R5 and X4 quasispecies by genosorting. This genosorting method is performed by using fluorochrome labelled R5 or X4-specific probes. After the binding of viral populations, the two viral phenotypes are separated by capillary electrophoresis. Probes with undetermined results and those with suspected mix or dual-tropic virus undergo a further step of replicative phenotyping. Phenotyping phase involves obtaining recombinant viruses possessing recombinant env sequence, derived from patient’s plasma sample. Recombinant virus replication occurs in cells expressing X4 or X4/R5, in the presence of some drugs, while differentiating dual-tropic viruses from mixed populations during the viral cycles. The replication of recombinant viruses is accomplished by three - four cycles, which takes about four days. This stage results in a large viral population with amplification of minority viral species and differentiation of dual-tropic viruses from mixed populations. The advantages are high sensitivity regarding the detection of minority variants and low turn-around time (Xtrack C). PhenX-R variants allow better detection of minority viral variants and separate dual tropic viruses from mixed tropism virus populations. Viral replication capacity provides additional information about viral fitness. The method is recommended to be used only by laboratories that have adequate security systems (16).

\textbf{Virco tropism platform (Virco BVBA, Mechelen, Belgium)}

The method involves four assays based on RT-PCR amplification of the variable region of gp120, located between areas V1 and V4 (NH2-V4). Each stage can be done separately, according to purpose:

- V3-population-based sequencing and determination of viral tropism;
- Analysis of NH2-V4 region by genotyping;
- NH2-V4-region analysis by phenotyping;
- Population-based NH2-gp 120-V4 phenotypic testing.

For phenotypic testing, NH2-V4 amplicons are cloned into an hXB2D backbone containing eGFP in \textit{nef} and deleted for HN2-V4 by in vitro recombination. Vector backbone pHXB2D \textit{ΔNh2V4-eGFP} and HN2-V4 amplicon of the patient sample is recombined in vitro in \textit{E. coli}. Recombinant plasmid DNA encoding the entire infectious genome is transfected in T-293 cells. Recombinant particles obtained are then used to infect indicator cells U87. These recombinant particles are able to infect U87-CD4 indicator cells expressing CD4 only or CD4 receptor and CXCR4 or CCR5 coreceptors. The ability to infect these indicator cells is evaluated by determining the level of expression of eGFP. Phenotypic analysis of viral tropism is then carried out by eGFP analysis using Argon laser microscope. One advantage is that it may reveal minority viral variants at a rate of 5% in samples with high viral load, but cannot distinguish between variants with dual tropism or mixed tropism (16).
**Genotypic Methods**

An alternative to identifying the coreceptor used by HIV-1 is genotyping, another technique which resorts to the amino acid sequences of region V3 of gp120, the major determinant of viral tropism (19, 20). Recently, as part of the 5th IAS Conference, Cape Town 2009, alternative diagnostic methods for testing HIV tropism were presented (21-23). Stucki et al (21), Harrigan et al (22) presented studies that use features of V3 region for interpretation of the viral tropism, beyond simple genotyping. Viral RNA is extracted from blood plasma. The amplification of V3 region (in triplicate with nested reverse transcriptase-PCR) are sequenced and analyzed using a software named RE Call. Sequences are then submitted to a bioinformatics algorithm such as geno2pheno to infer viral tropism from the V3 region. Sequences are inferred to be non-R5 if their geno2pheno false positive rate falls below 5.75%. If any one of the three sequences from a sample is inferred to be non-R5, the patient is unlikely to respond to a CCR5 antagonist (22, 23). Position-specific scoring matrices (PSSM) have been constructed to detect nonrandom distribution of amino acids at adjacent sites, with the potential to detect even relatively minor sequence changes that may have biological consequences on HIV-1 tropism antagonist. A series of genotyping algorithms were developed and they are available on websites (20).

Potential advantages of genotypic assays are increased sensitivity, low costs, shorter turnaround time, fewer sample failures, and providing information on viral clade (subtype) and drug resistance (23).

Currently, comparative studies examining the accuracy of genotypic and phenotypic assays for determining HIV tropism have been published. Low et al (24) evaluated the performance of several V3 algorithms using the plasma collected from 977 patients naive to antiretroviral therapy, previously tested with Trofile technique. The genotypic algorithms showed a high specificity of 88-97%. The sensitivity for detecting X4 variants was more modest, 22-45%. More recently, studies carried out on distinct populations using other phenotypic recombination tests registered sensitivity ranks superior to the genotypic tests used for detecting X4 variants, especially in patients experienced to the antiretroviral therapy and who were infected with subtype B. As a matter of fact, the genotyping techniques, through future improvements, seem to be easier and faster to use for determining tropism. Genotypic assays can be manipulated to have good specificity for identifying an X4 virus (7, 18, 20-23).

**Conclusions**

Viral entry represents one of the most attractive steps in the search for new drugs to treat HIV infection. CCR5 and CXCR4 are the major chemokine coreceptors used by HIV to efficiently enter into human cells. CCR5 antagonists have demonstrated efficacy both in treatment-experienced and treatment-naïve individuals. Maraviroc is the only approved CCR5 antagonist, which specifically inhibits the replication of R5 viruses. Virus tropism testing is crucial before prescribing CCR5 antagonists. The Trofile assay is only one of the phenotypic assays available for tropism testing, it has been used in clinical trials and is currently in routine use for testing HIV tropism in patients. Other assays, based on the genotypic or phenotypic methods, have been introduced in clinical use and some more continue to develop, in order to assess HIV strains coreceptor tropism.

**Abbreviations**

ARV: antiretroviral  
CCR5: cystein-cystein receptor 5  
CD4: cluster of differentiation 4  
CXCR4: cystein-X-cystein receptor 4  
DC: dendritic cell  
ECL2: second extracellular domain  
HAART: highly active antiretroviral therapy
HIV: human immunodeficiency virus
NSI: non-syncytium inducing
PMBC: peripheral mononuclear blood cells
SI: syncytium inducing

References

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