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The biological importance of Human Immunodeficiency Virus Type 1 (HIV-1) Circulating Recombinant Form (CRF) CRF02_AG

A dissertation submitted to the Faculty of Biological Sciences of the Vrije Universiteit Brussels in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in Biological Sciences.

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Finding the answer brings
Knowledge
Understanding the answer brings
Wisdom

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SUMMARY

The Human Immunodeficiency Virus (HIV) is very variable, as a consequence of high replication and mutation rates and, importantly, very frequent recombination. Of the two major "types" (HIV-1 and HIV-2), HIV-1, and more particularly "group M" viruses are responsible for the pandemic. Within HIV-1 group M, 9 "subtypes" or "clades" (A-K) and 18 "circulating recombinant forms" (CRF) can be distinguished. Even the individual patient virus shows increasing variability over the natural course, resulting in a swarm of different but related viral clones (quasispecies). Therefore each isolate can be considered as an individual "strain".

Homologous recombination of HIV occurs when a cell is infected with two different strains. Therefore, recombinant viruses were first observed in areas, where several HIV subtypes co-circulate in the population. Of the 18 circulating recombinants, mainly CRF01_AE, CRF02_AG and CRF03_AB are of epidemiological importance. CRF02_AG plays an important role in the epidemic in West Africa, where it is rapidly spreading and causes 31% of all new HIV-1 infections. This recombinant form was also reported in West Central and East African countries. This thesis will focus on diagnosis, fitness and prevention of this important recombinant

Our knowledge of the distribution of strains of HIV in different populations and about changes in that distribution over time is rather limited. This is because there have been few systematic large-scale attempts to characterize HIV isolates, and especially CRFs, emerging from different parts of the world. Sequencing is the gold standard of subtyping but due its high cost and difficulty to transfer to poor resource settings, there are low numbers of full-length genome sequences from many areas. Other subtyping methods are available e.g. Heteroduplex Mobility Assay (HMA), but the close genetic distance between CRF02_AG and subtype A strains hampered differentiation between CRF02_AG and subtype A by env HMA and additional experimental conditions are required with gag HMA.

In this thesis, we describe the design and potential use of a CRF02_AG-specific oligonucleotide probe hybridization assay for large-scale monitoring of the prevalence of CRF02_AG variants (**Chapter 3 – Section 3.1**). The newly developed oligonucleotide probe hybridization assay showed high signal reactions to probes, differentiating CRF02_AG from other subtypes (A to H) and CRFs (CRF01 and CRF06). The oligonucleotide probe hybridization assay was validated and had a sensitivity of 98.4%, a specificity of 96.7%, a positive predictive value of 98.4%, and a negative predictive value of 96.7%, which make the assay very reliable.

CRF02_AG has spread much more rapidly than other clades and other CRFs in West and West Central Africa. This raises concern that CRF02_AG may be favoured, in terms of superior replication fitness and/or transmission efficiency. In **Chapter 3 – Section 3.2** we tested the hypothesis that the predominance of CRF02_AG recombinants in West Central Africa is related to a higher replicative fitness. Therefore, we performed pair-wise competitions of a number of primary CRF02 strains with primary "pure" A and G strains, all sampled in the same area of Cameroon.

In order to mimic two important micro-environments, we performed the competitions in activated T cells (pathogenesis model) and dendritic cells (DC) (transmission model). In activated T-cells, direct dual infection/competition experiments clearly indicated that CRF02_AG isolates had a significant replication advantage over the "pure" subtype A and subtype G, and the higher fitness of CRF02_AG was evident for isolates from both AIDS and non-AIDS patients and was not dependent of biophenotype of the viruses (X4 or R5). In MO-DC cultures, CRF02_AG isolates showed a slightly higher but not significant replicative advantage. These results may explain the advantageous spreading of CRF02_AG variants in West and West Central Africa during the past decade.

In some parts of Africa, the incidence of HIV is alarmingly high in younger women (age 15-25). Even though effective preventive measures (condoms) exist they are often not a feasible option for women due the need of consent from male partners.

In view of this situation, a new focus in prevention is on the development of microbicides that, when applied topically, should substantially reduce transmission of HIV. An ideal microbicide should be able to block infection at the port of entry (genital tract or rectal mucosa) and before integration of the virus in the host cell DNA. Thus binding/fusion inhibitors, reverse transcriptase inhibitors (RTI) and integrase inhibitors could be potential candidates. Clinical trails are likely to be carried out in Africa were prevalence of HIV is high, hence it is important to know if candidate microbicide can equally inhibit relevant circulating HIV strains, including CRF02_AG.

This inspired us to study the inhibitory effect of various reverse transcriptase inhibitors (RTI) on cell-free HIV in co-cultures of dendritic cells and resting CD4 T cells, modelling early target cells during sexual transmission (**Chapter 3 – Section 3.3)**. The compounds tested included the second-generation non-nucleoside reverse transcriptase inhibitors (NNRTI) TMC-120 (dapivirine) and TMC-125 (etravirine), both belonging to the diarylpyrimidines (DAPY compounds). We focused on a "pre-treatment model" that mimics the scenario where a drug acts on the virus before it can enter the epithelial layer and reach the subepithelial target cells (represented in our model by MO-DC and CD4 T cells). The six primary isolates (HIV-1 group M subtypes A, A/G, B, C and CRF02_AG) used in this study were mostly from seropositive African individuals and represent the relevant circulating subtypes. Primary HIV-1 group M isolates were similarly sensitive as the reference subtype B HIV-$1_{B\text{-}aL}$ to inhibition by NN-RTI. These data suggest that NN-RTIs inactivate RT enzymatic activity of different viral clades and might be proposed for further testing as a sterilizing microbicide worldwide.

Several in vitro studies have implied the use of second generation NNRTIs as possible topical microbicide. Gel formulations of second generation NNRTI UC781 (a thiocarboxanilide) and TMC120 are currently entering into Phase I clinical trials. Due to the small size and high lipophilicity of NNRTIs, it is possible that these molecules when used as topical microbicide would be absorbed through the mucosa.

In **chapter 3 – Section 3.4** we investigated the immune suppressive capacities of second generation NNRTIs on T cell responses to flu antigen, presented by monocyte derived dendritic cells (MO-DC). In parallel the antiviral activities of the NNRTIs were determined under continuous treatment of compound and the therapeutic index calculated. With prolonged exposure (7 days), TMC120 was the most immune suppressive followed by UC781 and TMC125. However the DAPY compounds had antiviral activity against HIV replication far below the immune suppressive concentrations. Clearly, the DAPY compounds tested have highly different immune suppressive profiles but show a similar wide range of therapeutic index in vitro.

In summary we developed and validated a simple oligonucleotide probe hybridization assay that will be useful to monitor the spread of CRF02 in the pandemic. We showed that CRF02 has a replicative advantage over the parental A and G strains, potentially explained the preferential spread of the recombinant in West-Central Africa amongst many co-circulating strains. Finally, we showed that pre-treatment of primary virus isolates of the most prevalent subtypes, including CRF02, with DAPY compounds, a novel class of NNRTI can effectively prevent infection of monocyte-derived dendritic cells and T4 cells in vitro. Antiviral activity was seen in the nanomolar range, well below the toxic or immune suppressive dose, providing a favourable perspective for development as microbicide.

SAMENVATTING

Het Humaan Immunodeficiëntie Virus (HIV) is erg variabel. Er wordt onderscheid gemaakt tussen twee grote types (HIV-1 en HIV-2), waarvan HIV-1, en meer bepaald de “groep M” virussen, wereldwijd verspreid zijn. Binnen HIV-1 groep M worden dan weer 9 subtypes (A-K) onderscheiden en 18 zogenaamde “circulerende recombinante vormen (CRF in het Engels). Maar de variabiliteit gaat tot op het individuele niveau: het isolaat van elke patient bestaat uit een unieke zwerm van verwante, maar toch verschillende virussen die samen een afzonderlijke quasispecies of stam vormen.

Homologe recombinatie van HIV kan voorkomen wanneer een cel geïnfecteerd wordt met twee verschillende stammen. Recombinante HIV stammen werden dan ook gevonden bij geïnfecteerde personen in gebieden waar meerdere genotypische varianten of subtypes co-circuleren. Slechts drie van de 18 circulerende recombinanten, CRF01_AE, CRF02_AG en CRF03_AB zijn van epidemiologisch belang. CRF02_AG speelt een belangrijke rol in de West Afrikaanse HIV epidemie: het is verantwoordelijk voor 31% van alle nieuwe infecties in die regio. Deze recombinant is ook gerapporteerd in West Centraal en Oost Afrika. CRF02_AG verspreid zich nu snel in West-Afrikaanse landen, waar deze epidemie tot voor kort slechts beperkt was.

Tot nu toe zijn er slechts weinig systematische en grootschalige pogingen gedaan om HIV isolaten volledig te karakteriseren. Daarom hebben we geen goed zicht op de verspreiding van HIV stammen, subtypes en recombinanten in verschillende menselijke populaties en over de evolutie van die verspreiding over de tijd. Sequenering is de gouden standaard om subtypes te bepalen, maar er zijn slechts weinig volledige sequenties vanuit bepaalde gebieden bekend omwille van de hoge kost en de moeilijkheid om deze techniek over te dragen naar ontwikkelingslanden. Er zijn andere subtyperingstechnieken beschikbaar zoals de “Heteroduplex Mobility Assay (HMA)”, maar de geringe genetische afstand tussen de subtype A fragmenten in subtype A zelf en in CRF02_AG bemoeilijkt de toepassing van *env* HMA in dit geval.

In deze thesis beschrijven we de ontwikkeling en het potentiële gebruik van een CRF02-specifieke "oligonucleotide probe hybridisation assay" voor de monitoring van de prevalentie van CRF02 varianten op grote schaal. (**Hoofdstuk 3 –Sectie 3.1).** Deze nieuwe oligonucleotide probe hybridisation assay kon duidelijk CRF02_AG van andere subtypes (A-H) en CRFs (CRF01-CRF06) onderscheiden. De test had een sensitiviteit van 98.4% , een specificiteit van 96.7%, een positieve predictieve waarde van 98.4% en een negatieve predictieve waarde van 96.7%. We kunnen dus stellen dat het om een zeer betrouwbare test gaat.

CRF02_AG heeft zich in West- en West-Centraal Afrika dus veel sneller verspreid dan andere subtypes en CRFs. Dit gegeven doet vermoeden dat CRF02_AG bevoordeeld zou kunnen zijn in termen van "replicatieve fitness" (snelheid van vermenigvuldiging) en/of transmissie efficiëntie. In **Hoofdstuk 3 –Sectie 3.2** hebben we de hypothese getest dat het overwicht van de CRF02 recombinanten in deze regio gerelateerd is aan een hogere replicatieve fitness. Om dit na te gaan, voerden we paarsgewijze competities uit tussen een aantal primaire CRF02 stammen en primaire "zuivere" isolaten van subtype A en G, die allen afkomstig waren van dezelfde streek in Kameroen. Om twee belangrijke micro-milieus na te bootsen, voerden we competities uit in geactiveerde T cellen, als model voor pathogenese en in dendritische cellen (DC) als "transmissie model". In geactiveerde T cellen toonden directe paarsgewijze infectie-competitie experimenten duidelijk aan dat CRF02 isolaten een significant replicatief voordeel hebben over de "zuivere" subtype A en G stammen. De grotere "fitness" van CRF02_AG was duidelijk voor isolaten van zowel AIDS patiënten als van personen met een minder gevorderd ziektebeeld. De hogere fitness was niet afhankelijk van het virale biofenotype (R5 of X4). In monocyt-afgeleide DC vertoonden de CRF02 isolaten ook een lichtjes (maar niet echt significant) hogere fitness. Deze resultaten zouden de toegenomen verspreiding van CRF02_AG varianten in West en West Centraal Afrika gedurende de laatste tien jaar kunnen verklaren.

In sommige delen van Afrika is de incidentie van HIV vooral alarmerend hoog in jonge vrouwen (tussen 15 en 25 jaar).

Alhoewel er effectieve preventieve middelen (condooms) bestaan, zijn ze geen haalbare optie voor vele vrouwen omdat ze medewerking van de mannelijke partner vereisen. Daarom is er een nieuwe focus in de preventie, namelijk het ontwikkelen van microbiciden. Het gaat per definitie om middelen, die bij locaal gebruik de transmissiekans van HIV drastisch moeten verminderen. Een ideaal microbicide zou de infectie moeten blokkeren aan de ingangspoort (de genitale tractus of de rectale mucosa) en op cellulair niveau in alle geval de integratie van het virus in het DNA van de gastheercel moeten voorkomen. In principe komen daarom inhibitoren van binding, fusie, reverse transcriptase en integrase in aanmerking.

Klinische microbiciden trials zullen uitgevoerd worden in Afrika, waar de prevalentie van HIV hoog is. Daarom is het belangrijk dat kandidaat microbiciden actief zijn tegen relevante circulerende stammen, waaronder CRF02_AG. Vandaar ook dat we het inhiberende effect van verschillende reverse transcriptase inhibitoren (RTI) op cel-vrij virus bestudeerden in een co-cultuur van dendritische cellen en rustende CD4 T cellen, als model van vroege doelwitcellen tijdens sexuele transmissie (**Hoofdstuk 3 – Sectie 3.3)**. De geteste molecules omvatten de tweede generatie non-nucleoside reverse transcriptase inhibitoren (NNRTI) TMC-120 (Dapivirine) en TMC-125 (Travertine), beide behorend tot de zogenaamde DAPY (di-aryl pyrimidine reeks).

We concentreerden ons op een “voorbehandelingsmodel”, waarin het scenario wordt nagebootst van een geneesmiddel dat op het virus inwerkt vooraleer dit de epitheliale laag kan binnendringen en de sub-epitheliale doelwitcellen bereiken (die in ons model dus vertegenwoordigd worden door de DC en CD4 T cellen). De zes primaire isolaten van HIV-1 groep M subtypes A, AG, B, C en CRF02, die in deze studie gebruikt werden, waren overwegend van Afrikaanse patiënten en vertegenwoordigen de meest relevante circulerende subtypes. Infectie van de DC/T co-culturen kon bij een gelijkaardige dosis van de NNRTI voorkomen worden, onafhankelijk van welke primaire stam gebruikt werd. Deze gegevens tonen aan dat de geteste NNRTIs de reverse transcriptase van verschillende subtypes even efficiënt blokkeert en dat ze dus kunnen voorgesteld worden om verder te testen met het oog op de ontwikkeling van HIV-steriliserende microbiciden.

Verscheiden in vitro studies hebben reeds argumenten gegeven voor het gebruik van NNRTI als mogelijke microbiciden. Gel formuleringen van de tweede generatie NNRTI UC781 (behorend tot de thiocarboxanilides) en TMC120 (DAPY) gaan op dit ogenblik in fase I trial. Omwille van de kleine omvang en het sterk lipofiele karakter van NNRTIs is het mogelijk dat deze molecules, wanneer ze gebruikt zouden worden als microbicide, geabsorbeerd zouden worden doorheen de mucosa. In **hoofdstuk 3 – Sectie 3.4** onderzochten we de immuno-supressieve eigenschappen van de tweede generatie NNRTI op het opwekken van T cel responsen t.o.v. influenza antigenen, gepresenteerd door DC. De anti-virale activiteit van de NNRTI in continue behandeling werd tegelijk in parallel bepaald. Gebaseerd op deze twee gegevens werd een therapeutische index berekend. Bij langdurige behandeling (7 dagen) was TMC120 het meest immuunsuppressief, gevolgd door UC781 en TMC125. Echter, beide DAPY compounds hadden een antivirale activiteit bij concentraties, die ver beneden de immunosuppressieve concentraties liggen. M.a.w. hoewel de DAPY compounds een sterk verschillende immunotoxische drempel hebben in vitro, is de therapeutische index voor beide heel hoog.

Samenvattend: deze thesis focuste op diagnose, fitness en preventie van HIV-1 CRF02_AG. We hebben een relatief eenvoudige oligonucleotide probe hybridisatie assay ontwikkeld en gevalideerd, die bruikbaar zal zijn voor het monitoren van de verspreiding van CRF02 in de pandemie. We toonden aan dat CRF02 een replicatief voordeel heeft op de parentale A en G stammen. Deze eigenschap kan de preferentiële verspreiding van deze recombinant in West-Centraal Afrika temidden van allerlei co-circulerende stammen verklaren. Tenslotte toonden we aan dat voorbehandeling van primaire virus isolaten van de meest prevalente stammen (waaronder CRF02) met NNRTI DAPY compounds in vitro effectief de infectie van monocyt-afgeleide DC en T4 cellen kan voorkomen. De antivirale activiteit werd vastgesteld in de nanomolaire range, ver beneden de toxische en immuno-suppressieve dosis, hetgeen een gunstig perspectief biedt voor hun ontwikkeling als microbicide.

THESIS OUTLINE

This thesis reports on the diagnosis, biological importance and prevention of Human Immunodeficiency Virus Type 1 (HIV-1) Circulating Recombinant Form (CRF) CRF02_AG.

Chapter 1 gives a general introduction to Human Immunodeficiency Virus Type 1 (HIV-1) a member of the retroviridae family. The epidemiological importance of CRF02_AG is highlighted and the rationale for studying the recombinant virus.

Chapter 2 describes in detail the laboratory techniques used in this study.

Chapter 3 reports on the results of the specific objectives of this study

> **Section 3.1** describes the development a probe hybridization assay for identifying Human Immunodeficiency Virus Type 1 (HIV-1) Circulating Recombinant Form (CRF) CRF02_AG.
>
> **Section 3.2** explores the hypothesis that the predominance of Human Immunodeficiency Virus Type 1 (HIV-1) Circulating Recombinant Form (CRF) CRF02_AG in West Central Africa is related to a higher replicative fitness.
>
> **Section 3.3** evaluates the inhibitory effect of various reverse transcriptase inhibitors (RTI) on relevant cell-free HIV isolates (including HIV-1 CRF02_AG) in co-cultures of dendritic cells and resting CD4 T cells, modeling early target cells during sexual transmission: Implications for use as microbicide worldwide.
>
> **Section 3.4** investigates the immune suppressive capacities of second generation NNRTIs on T cell responses to flu antigen, presented by monocyte derived dendritic cells (MO-DC).

Chapter 4 discusses the findings presented in this thesis and provides perspectives for future research.

LIST OF ABBREVIATIONS

A	:	Adenine
AA/BB	:	Acrylamide / Bisacrylamide
Ab	:	Antibody
AIDS	:	Acquired Immunodeficiency Syndrome
AP	:	Alkaline Phosphatase
APS	:	Ammonium Persulphate
ART	:	Antiretroviral Treatment
ATP	:	Adenosine triphosphate
AZT	:	Azidothymidine
bp	:	Base pair
BSA	:	Bovine Serum Albumin
C	:	Cytosine
CA	:	Capsid
CD4	:	Cluster of Differentiation 4
cDNA	:	Complementary DNA
CO_2	:	Carbon dioxide
cpm	:	Counts per minute
cpx	:	Complex
CRF	:	Circulating Recombinant Form
CS	:	Cellulose-Sulphate
DAPY	:	Diarylpyrimidine
DC	:	Dendritic Cells
DC-SIGN	:	Dendritic Cell Specific ICAM-3 Grabbing Non-Intergrin
DEPC	:	Diethylpyrocarbonate
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxynucleotide Triphosphates
ds	:	Double Stranded
EDTA	:	Ethylenediamine Tetra Acetic Acid
Env	:	Envelope
ER	:	Endoplasmic Reticulum

FACS	:	Fluorescent Activated Cell Sorter
FBS	:	Fetal Bovine Serum
FGT	:	Female Genital Tract
FITC	:	Fluorescein-Iso-Thio-Cyanate
G	:	Guanine
gag	:	Group Antigen
gp	:	Glycoprotein
gp120	:	Surface Envelope Glycoprotein
gp160	:	Large Precursor Polyprotein
gp41	:	Transmembrane Glycoprotein
group M	:	Major Group
group N	:	Non-M / Non-O group
group O	:	Outlier group
GuSCN	:	Guanidine thiocyanate
H^+ ions	:	Hydrogen ions
H_2O	:	Water
HAART	:	Highly Active Antiretroviral Therapy
HCl	:	Hydrogen Chloride
HIV	:	Human Immunodeficiency Virus
HIV-1	:	Human Immunodeficiency Virus Type 1
HIV-2	:	Human Immunodeficiency Virus Type 2
HMA	:	Heteroduplex Mobility assay
HPLC	:	High Pressure Liquid Chromatography
HR1	:	Heptad Repeat 1
HR2	:	Heptad Repeat 2
HTA	:	Heteroduplex Tracking Assay
HuMAb	:	Human Monoclonal Antibody
HuS	:	Human Serum
ICAM-3	:	Intercellular Adhesion Molecule 3
IDU	:	Intravenous Drug Users
IL	:	Interleukin
IN	:	Integrase

IRF	:	Intersubtype Recombinant Forms
Kb	:	Kilo base
KD	:	Kilo Dalton
LTNP	:	Long term non progressors
LTR	:	Long Terminal Repeat
MA	:	Matrix Protein
$MgCl_2$	:	Magnesium Chloride
$MgSO_4$	:	Magnesium Sulphate
MHC	:	Major Histocompatibility Complex
MO-DC	:	Monocyte derived Dendritic Cells
MOI	:	Multiplicity Of Infection
mRNA	:	Messenger Ribonucleic acid
MTP	:	Micro Titre Plates
N-9	:	Nonooxynol-9
Na_2HPO_4	:	Di Sodium hydro phosphate
NaH_2PO_4	:	Sodium Di hydro phosphate
NaOH	:	Sodium Hydroxide
NC	:	Nucleocapsid
Nef	:	Negative regulator Factor
NN-RTI	:	Non-Nucleoside Reverse Transcriptase Inhibitors
N-RTI	:	Nucleoside Reverse Transcriptase Inhibitors
NSI	:	Non-Syncytia Inducing
Nt-RTI	:	Nucleotide Reverse Transcriptase Inhibitors
OD	:	Optical Density
PAGE	:	Polyacrylamide gel
PBMC	:	Peripheral Blood Mononuclear Cells
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PHA	:	Phytohaemagglutinin
PI	:	Protease Inhibitor
PLL	:	Poly - L - Lysine
pNPP	:	para-nitrophenylphosphate

Pol	:	Polymerase
PR	:	Protease
Rev	:	Regulator of Viral protein expression
RNA	:	Ribonucleic Acid
RNase A	:	Ribonuclease A
RNase H	:	Ribonuclease H
RRE	:	*rev* responsive element
RT	:	Reverse Transcriptase
RTI	:	Reverse Transcriptase Inhibitor
RT-PCR	:	Reverse Transcriptase PCR
SDS	:	Sodium dodecyl sulphate
SI	:	Syncytia-Inducing
SiO_2	:	Silicon Dioxide
SIV	:	Simian Immunodeficiency Virus
SIVcpz	:	Chimpanzee related Simian Immunodeficiency Virus
SIVmac	:	Macaques related Simian Immunodeficiency Virus
SIVmn	:	Sooty Mangabeys Simian Immunodeficiency Virus
SSC	:	Saline Sodium Citrate
T	:	Thymidine
TAE	:	Tris Acetate EDTA
Taq	:	Thermos Aquaticus
Tat	:	Trans-Activator of Transcription
TBE	:	Tris Boric EDTA
$TCID_{50}$	:	Tissue Culture Infecting Dose
T_d	:	Dessociation temperature
TEMED	:	N, N, N', N' - Tetramethylethylenediamine
TRIS	:	Tris Hydroxyl Methyl Amino Methane
URF	:	Unique Recombinant Form
UV light	:	Ultra Violet light
Vif	:	Viral Infectivity Factor
Vpr	:	Viral Protein R
Vpu	:	Viral Protein U

1.1 History of Acquired Immune Syndrome (AIDS)

AIDS (Acquired Immune Syndrome) is a disease complex characterized by gross functional impairment of the immune system as a consequence of infection by the Human Immunodeficiency Virus Type 1 (HIV-1) and/or Human Immunodeficiency Virus Type 2 (HIV-2), two significantly different retroviruses.

HIV-1 and HIV-2 are the second known human representatives of the lentiviruses, a subfamily of retroviruses. HIV-1 was first isolated in 1983 [1], and its links as cause of AIDS reported in 1984 [2, 3]. In 1986, Clavel and colleagues isolated a new closely related but distinct retrovirus associated with clinical conditions of AIDS from two HIV-1 seronegative West African individuals with AIDS. This new virus was named HIV-2 [4].

HIV-1 is responsible for the global pandemic, while HIV-2 until recently has been restricted to West Africa and appears to be less virulent in it effects. A global estimate of adults and children living with HIV/AIDS at the end of 2004 is approximately 40 million, 75% of these are from Sub Saharan Africa [5].

1.2 Structure of HIV

The HIV virion has a diameter of 80-100nm, it is surrounded by a lipid bilayer, that is cellular in origin. Each virion expresses 72 glycoprotein projections composed of gp120 and gp41 (*figure 1*). gp41 is a transmembrane molecule that crosses the lipid bilayer of the envelope. gp120 is noncovalently associated with gp41 and serves as viral ligand for the receptors on host cells. The viral envelope also contains some host cell membrane proteins such as class I and class II MHC molecules. Within the envelope there is a viral core, or nucleocapsid, which includes an outer layer, composed of a protein called p17 and an inner layer, composed of the protein, called p24.

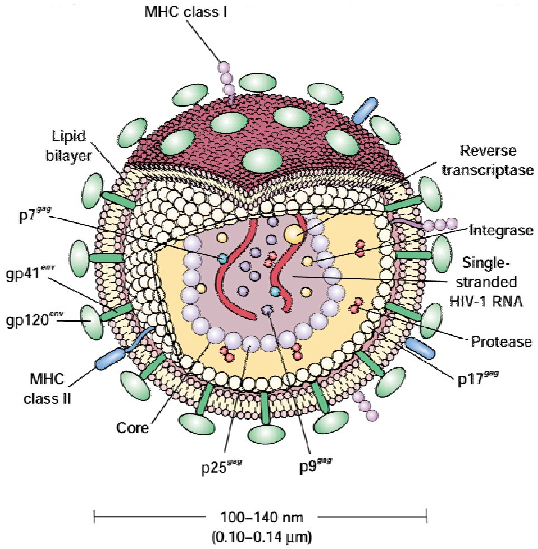

Figure 1: Structure of HIV-1 [6]

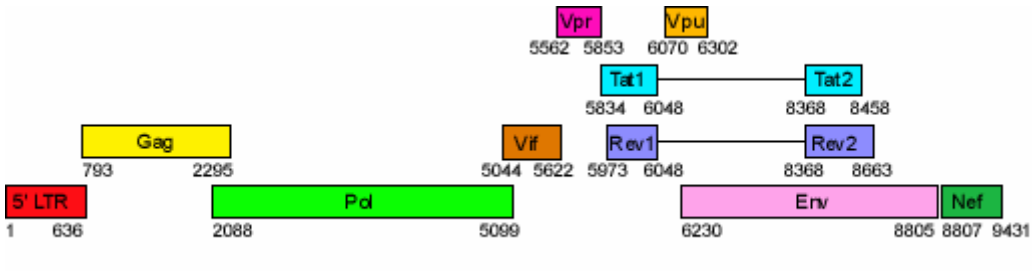

Figure 2: The HIV-1 genome [7].

The HIV genome is approximately 9.7kb in size (*figure 2*) and exists within the virion as a dimer with two identical (+) RNA strands [8], which are associated with two molecules of reverse transcriptase p64 and nucleoid proteins p10, a protease, and p32, an integrase.

Each RNA strand has a copy of the virus's nine genes. Three of these, gag, pol, and env are structural genes. Another three, tat, rev and nef are regulatory genes. The last three vif, vpr, and vpu are accessory genes [9]. The three structural genes, gag, env and pol present in all retroviruses, encode proteins required for production of infectious progeny virions.

The primary product of the gag gene is a precursor protein, which is cleaved after translation to form nucleocapsid products, mainly a 24-KD protein p24. The pol gene encodes the highly conserved enzymatic proteins, protease, reverse transcriptase (RT), integrase and ribonuclease H. The env gene encodes a glycosylated precursor protein gp160, which is cleaved to produce an external glycoprotein gp120 involved in recognition and binding to target cell receptors, and a smaller hydrophobic transmembrane protein gp41 involved in membrane fusion.

Non structural genes include accessory and regulatory genes: vif, vpr, vpu, nef, tat & rev. These genes are encoded in six overlapping open reading frames, which are well conserved. Vif protein facilitates virion assembly and maturation and is important for production of highly infectious mature virions [10]. Viruses with the vif gene deleted can replicate normally in certain “permissive” cell lines, while infection of “non permissive” cells produce only defective virions. Genetic evidence suggested that a factor in non-permissive cells called APOBEC3G [11] inhibits propagation of the virus, but this factor is normally inactivated by the Vif gene. Vpr protein arrests growth of infected cells in the G2 phase and targets reverse transcriptase, integrase, RNA and matrix protein to the nucleus [10].

Vpu protein is present only in HIV-1 and promotes CD4 and MHC class I degradation in the endoplasmic reticulum [12], Nef protein, degrades surface CD4 and MHC class I to enhance env incorporation into virions. It is also involved in enhancing viral infectivity [10, 12]. Tat protein increases the initiation of transcription by binding to viral LTR sequence and activating transcription of all viral genes. Rev protein regulates splicing and transport of viral RNA by binding to an un-spliced RNA at a longer stem loop structure known as the rev responsive element (RRE), located in the env gene [10, 12].

1.3 Viral life cycle

HIV infection of a susceptible host cell (*figure 3*) begins when gp120 (the glycoprotein which forms the spikes sticking out of a HIV virus particle) binds to the CD4 receptor on the host cell. CD4 is expressed on the surface of the T-helper lymphocyte subset and, in lesser quantities on mononuclear phagocytes; these are the principle target in vivo [13]. Alternative routes of entry besides CD4 have been identified, but their biological significance is uncertain [14]. Later studies indicated that a coreceptor is needed for HIV to enter the cell [15-17]. The major co-receptors are the chemokine receptors CXCR4 and CCR5.

Following binding to the surface of the host cell, a pH-independent fusion of viral and cell membrane occurs, and the viral core is taken up into the cytoplasm [18]. The genetic material of the virus, which is RNA, is released and undergoes reverse transcription into DNA. The HIV enzyme reverse transcriptase (RT) is necessary to catalyze this conversion of single-stranded viral RNA into double-stranded DNA. The kinetics of reverse transcription is more rapid in CD4+ lymphocytes than macrophages, which suggest that cellular factors are also involved [19].

Once the genetic material of HIV has been changed into DNA, this viral DNA is translocated to the nucleus and integrated to the host genome by collinear insertion into chromosomal DNA to form the provirus. The enzyme integrase catalyzes this process. Successfully integrated proviral DNA may remain silent and latent until the host cell is activated. The provirus is used as template to produce viral genomic RNA for progeny viral particles and mRNA for translation to produce viral proteins. The HIV protease cleaves precursor poly-proteins into the right sizes. Virions are then assembled in the cytoplasm and are released from the cell by budding through the cell surface membrane [20].

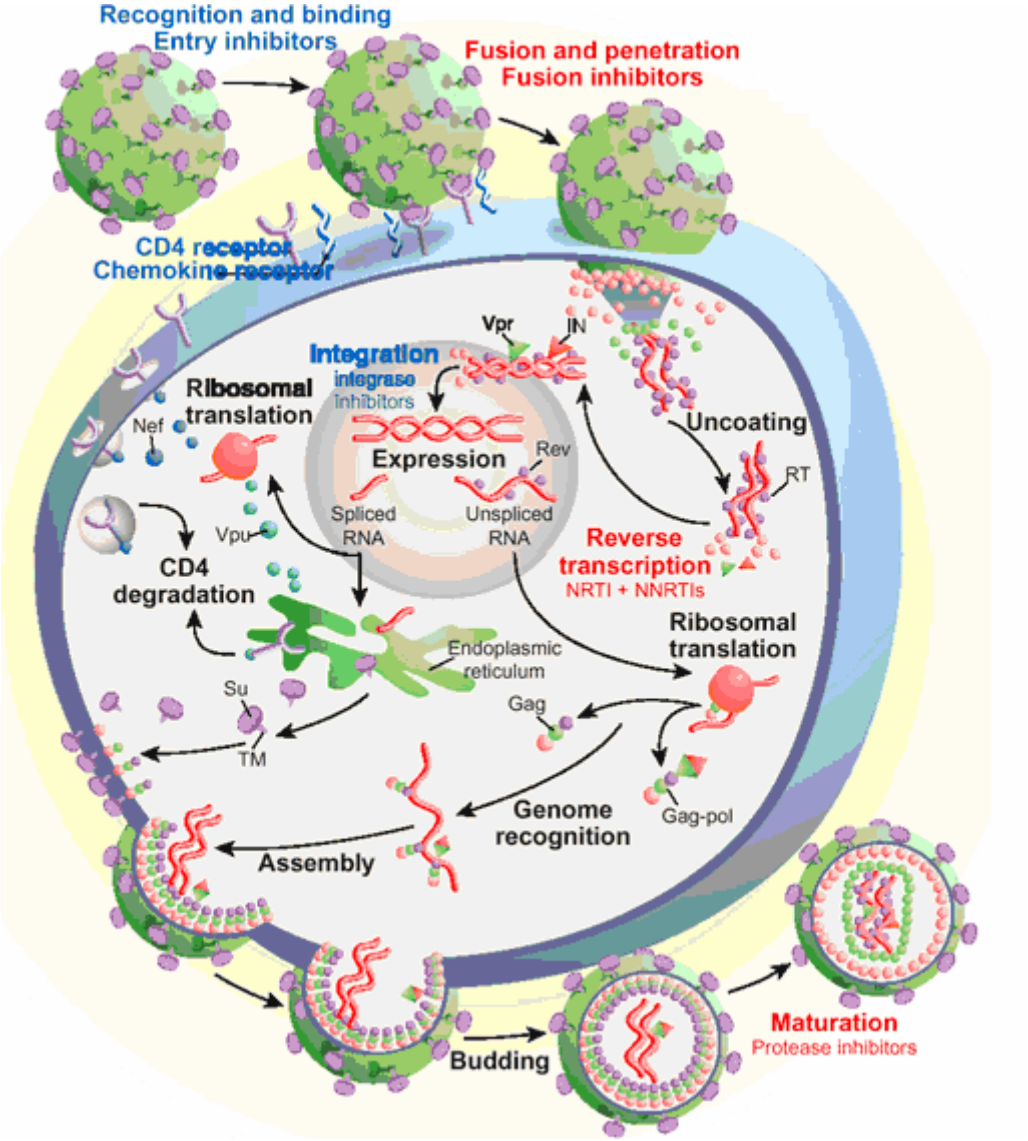

Figure 3: HIV life cycle [21]

1.4 Sexual Transmission of HIV

Heterosexual transmission of HIV accounts for 80% of the 40 million people living with the HIV virus [5]. Sexual transmission is mediated by exposure to infectious HIV-1 and/or infected cells in the semen or mucosal secretions. The relative transmissibility of cell-free versus cell-associated virus is still uncertain [22]. During sexual transmission HIV particles crosses the mucosal barrier of the genitourinary tract to reach the subepithelial residing target cells [23, 24].

The exact mode of transmission is not certain and multiple mechanisms have been proposed [25, 26]. Most importantly it was demonstrated that the primary targets of simian immunodeficiency virus (SIV) infection, following intravaginal infection of macaques, are cervical and vaginal subepithelial cells. Within 24-72 h after vaginal inoculation, vial DNA and RNA was detectable in dendritic cells (DC), intraepithelial lymphocytes and CD4+ T cells in the endocervical submucosa [27, 28].

Dendritic cells (DC) are the principle antigen presenting cells (APC) involved in primary immune responses; their major function is to obtain antigen (Ag) in tissues, migrate to lymphoid organs, and activate T cells. DCs are also the first immune cells to arrive at sites of inflammation on mucous membranes, the major site of sexual transmission of HIV. CD4 and CCR5 receptors found on the surface of DC allow both mature and immature DCs to establish productive HIV infection [29]. In addition DC-SIGN found on mature DCs do not function as a receptor for viral entry but efficiently captures HIV-1 in the periphery and facilitates its transport to secondary lymphoid organs rich in T cells, to enhance infection in trans of these target cells [30-32].

1.5 Genetic Variation

The occurrence of different genetic subtypes and recombinants as well as different phenotypes is only the tip of the iceberg of the tremendous genetic flexibility of HIV. The virus can alter structure, function and immunogenicity of its gene products in biologically important ways. No two HIV strains are the same, and even within a single individual, HIV is present as a 'quasispecies' – a pool of closely related but genetically distinct HIV variants [33, 34].

HIV genetic variation is due to its intrinsically high mutation and recombination rate. The frequent mutations are caused by the infidelity of reverse transcription (viral RNA to DNA) by the RT enzyme. The error rate of HIV RT has been estimated to be 0.3 error / replication in vivo [35, 36]. In addition, the RT very frequently “jumps” from one RNA strain to the other.

Therefore each proviral DNA is in fact a recombinant, but this will only be evident if two viral genomes from different strains were present in the particle, as a consequence of a dual cellular infection [36, 37]. Genetic variation is believed to be a mechanism by which the virus responds to in vivo selection pressure and is responsible for the rapid generation of drug resistance [38, 39] and variants that escape immune surveillance in infected individuals [40].

1.5.1 Mechanisms of producing viral variation

Both a mutation-prone reverse transcriptase and subsequent recombination between differing genomes are thought to contribute to the ability of retroviruses to generate viral diversity.

1.5.2 Reverse Transcriptase enzyme

HIV RT is a simple enzyme as compared to DNA polymerase consisting of a heterodimer of p66/p51 subunits enzyme. It posses two enzymatic functions located on different domains of the protein, a reverse transcriptase activity and a Ribonuclease H activity (RNase H) [41, 42]. Conversion of viral genomic RNA to double stranded DNA is a two-step process. The minus DNA strand is synthesized by using viral RNA as template. The cellular polymerase then uses the newly synthesized complementary DNA as a template for the second strand synthesis. The RNase H activity associated with RT digests the RNA in the first RNA:DNA hybrid.

RT lacks 3' - 5' exonuclease proof reading activity, hence wrong nucleotides incorporated in a growing chain are not corrected. The error rate of RT is 1000 times higher than DNA polymerase and is estimated to be at a rate of 1 error / replication cycle [43]. Mutations, as a result of RT errors include not only point mutations but also deletions, insertion, duplication and frame shifts.

The majority of point mutations are considered silent i.e. they do not produce phenotypic consequences to the viral offspring. These “synonymous” mutations usually occur at the third base, which is “promiscuous”: a different third nucleotide most of the time does not result into an amino acid substitution ("wobble effect"). Moreover, in the cases where an amino acid substitution is observed (non-synonymous mutation) it doesn't necessarily infer phenotypic consequences, depending on the nature and location of new amino acids in the protein [44]. Sometimes a virus might benefit from a non-silent (non-synonymous) mutation e.g. when drug resistant isolates are formed or mutants that escape immune surveillance.

1.5.3 Recombination

The presence of a dimeric RNA genome and the capacity of the RT to shift or switch templates during transcription makes HIV and other retroviruses highly recombinogenic [37]. Recombination takes place during the synthesis of both the minus strand and the plus strand DNA prior to integration in the host genome [45]. Coffin [46] proposed the modified copy – choice model, in which RT switches from one RNA template to another upon encountering breaks in the RNA strand - intermolecular recombination.

When the viral RNA genomes is not damaged, the alternative model, proposed by Junghans et al [47], assumes that the two RNA genomes are each reverse transcribed into negative-strand DNA and that single-stranded DNA branches are formed and recombine with homologous regions on the other cDNA in a displacement-assimilation mechanism -intramolecular transfer and plus strand recombination occurs.

Strand transfers occur more frequently when the two strands are identical or very closely related. If the other strand is non-homologous compared to the first one, recombination can occur, but the rate is very low compared to homologous recombination [48].

The surprising high frequency in mosaic HIV-1 sequences (Circulatory Recombinant Forms, CRFs, documented in several independent individuals and Isolated Recombinant Forms IRF – only documented in one person) in the database implies that a substantial proportion of individuals can become co-infected with HIV-1 strains belonging to different sequence subtypes, and that recombination between these genomes can occur in vivo to generate biologically active viruses, often with hybrid gag and env proteins [37].

The first HIV-1 strain to be identified as a recombinant was MAL from the Democratic Republic of Congo [49]. MAL is a recombinant virus including regions that are clearly of subtype A and D origin, there is a long region encompassing the 5' half of the pol gene that has defied characterization [37]. Now it appears that part of that region may be subtype K or CRF04_cpx [50]. Z321B isolated in 1979, from the Democratic Republic of Congo, is the oldest known hybrid virus. It appears to include sequence blocks from three (or four) different subtypes, A/G/I? /E? [51].

Currently there are 18 circulating recombinant forms of HIV-1 described; CRF01_AE, CRF02_AG, CRF03_AB, CRF04_cpx, CRF05_DF, CRF06_cpx, CRF07_BC, CRF08_BC, CRF09_cpx, CRF10_CD, CRF11_cpx, CRF12_BF, CRF13_cpx, CRF14_BG, CRF15_01B, CRF16_A2D, CRF17_cpx and CRF18_cpx. However the impact of each CRF on the global AIDS epidemic is vastly uneven. Of the 16 CRFs, only three are of epidemiological importance i.e. CRF01_AE, CRF02_AG (*figure 4*) and CRF03_AB.

Figure 4: Mosaic structure of Human Immunodeficiency Virus Type 1 (HIV-1) Circulating Recombinant Form (CRF) CRF02_AG [7].

1.6 Classification of HIV

HIV viruses are closely related to Simian Immunodeficiency Virus (SIV). HIV-1 shares a higher sequence homology with SIV of the Chimpanzee SIVcpz, than HIV-2, which in turn is more related to SIV of Sooty Mangabey SIVsm. [52]. A lot of evidence indicates that HIV viruses were introduced to humans from monkeys by cross species transmission, most probably on several separate occasions [53, 54].

1.6.1 Genotypic classification

HIV-1 and HIV-2 are related but differ about 50-65% on the nucleotide level. HIV-1 is phylogenetically divided in three groups: M (Main), N (non-M/ non-O) [55] and O (Outlier) [56]. Phylogenic analysis of the gag and env genes reveals 9 clades or subtypes within the HIV-1 Group M: A, B, C, D, F, G, H, J and K. These subtypes differ from each other by 10 – 30 % their distribution shows geographical variation.

The Circulating Recombinant Forms (CRFs), CRF01_AE, CRF02_AG, CRF03_AB, CRF04_cpx, CRF05_DF, CRF06_cpx CRF07_BC, CRF08_BC, CRF09_cpx, CRF10_CD, CRF11_cpx, CRF12_BF, CRF13_cpx, CRF14_BG, CRF15_01B, CRF16_A2D, CRF17_cpx and CRF18_cpx represent recombinant HIV-1 genomes that have been isolated from two or more persons who are not epidemiologically related, so that they can be assumed to have an epidemiologically relevant contribution to the HIV epidemic. The CRFs share an identical mosaic structure.

In the HIV-1 group O, subtypes are not yet clearly defined and agreed upon. The diversity of sequences within the HIV-1 group O is nearly as great as the diversity of sequences in the HIV-1 group M, but a phylogenic analysis of the gag and env genes does not reveal clades of viruses as clear as the clades detected in the HIV-1 group M. Subtypes are not yet defined for HIV-1 Group N; very few isolates have been identified.

As with HIV-1; the grouping of HIV-2 isolates into different sequence subtypes is generally consistent among trees derived from different genomic regions. That is virus that belongs to a particular lineage in env; also clusters within the same lineage in gag. Hence HIV-2 is phylogenetically divided into six subtypes: A, B, C, D, E and F. HIV-2, HIV-1 O and N are restricted to West-Central Africa and appear to be less virulent than HIV-1 group M in it effects.

1.6.2 Phenotypic classification

HIV-1 isolates fall into two distinct groups depending on their biological properties. Asjo et al [57] described the two groups as slow-low and rapid-high depending on their replication rates in peripheral blood mononuclear cells (PBMCs). Related reports described the two virus groups as nonsyncytium-inducing (NSI) or syncytium-inducing (SI) [58] as well as macrophage-tropic (or M-tropic) and T-cell tropic (or T-cell line tropic, T-tropic) [59]. Later, it was shown that the differences between the two types of isolate were determined by gp120 *env* sequences and were located predominantly in the V3 loop [60].

The puzzle of HIV phenotypes was further unravelled when E. A. Berger and colleagues cloned the first HIV coreceptor, CXCR4 (termed fusin) [15]. Coexpression of CXCR4 with CD4 on mouse cells conferred fusion by SI or T-tropic (but not NSI/M-tropic) HIV-1 strains. During the same year, several groups reported CCR5 as the coreceptor for NSI viruses [61-63]. Both CCR5 and CXCR4 are members of the 7TM chemokine receptor family. More than a dozen other 7TM receptors have been shown to act as coreceptors on CD4+ cell lines for particular HIV-1 strains. These coreceptors are also chemokine receptors or are closely related orphan receptors.

In summary, CCR5 and CXCR4 are the major HIV-1 coreceptors and all strains can use one (R5 or X4 viruses) or both (R5 and X4 viruses) to enter CD4+ cells. R5 viruses are predominantly transmitted and persist throughout infection. Viruses that exploit CXCR4 emerge late in disease and can be isolated from up to 50 % of AIDS cases. Currently, there is little evidence to suggest that coreceptors other than CCR5 and CXCR4 are used significantly *in vivo*.

1.7 Diagnosis of HIV

Diagnosis of HIV infection has been made on a serological basis because the majority of the course of infection is clinically undetectable.

1.7.1 Enzyme-linked Immunosorbent Assays (ELISA)

ELISA are the most commonly used tests to screen for HIV infection because of their relatively simple methodology, inherent high sensitivity, and suitability for testing large numbers of samples, particularly in blood testing centers. More than 40 different ELISA test kits are currently available, but only about 10 are licensed by the FDA for use in the United States [64]. A common feature of all varieties of ELISA is the use of enzyme conjugates that bind to specific HIV antibody, and substrates/chromogens that produce colour in a reaction catalyzed by the bound enzyme conjugate.

Several indirect ELISA incorporate polyvalent conjugates (anti-IgG and IgM) in order to increase sensitivity for detecting early infection (during seroconversion). The most recent addition to ELISA technology is the antigen sandwich method in which an enzyme (alkaline phosphatase or horseradish peroxidase) is conjugated to an HIV antigen (similar to the immobilized antigen on the solid phase). The antibody in the sample is "sandwiched" between two antigen molecules, one immobilized on the solid phase and one containing the enzyme. Subsequently, the addition of substrate results in colour development in proportion to antibody concentration. The antigen sandwich ELISA is considered the most sensitive screening method, given its ability to detect all isotypes of antibody (including IgM) [64]. One disadvantage of this method is the relatively large volume (150 µl) of sample required, which may make repeat testing and testing of samples from infants difficult.

1.7.2 Heteroduplex Mobility Assay (HMA)

This phenomenon of differential Heteroduplex migration was first exploited to detect and characterize allelic variation and mutations, particularly of inherited genetic disorders of humans [65-67]. The assay has also been used in several viral infections including measles [68], polio [69] and influenza [70].

HMA based on *env* and *gag* genes, classify in respective subtypes HIV-1 group M isolates [71-75], HMA has been evaluated as a reliable alternative subtyping method compared to sequencing and phylogenetic analysis [72, 75] and is sensitive, cost-effective, and applicable on a relatively large scale. It has already been successfully introduced by UNAIDS in several developing countries.

The Heteroduplex Mobility Assay (HMA) is a polymerase chain reaction (PCR) based technique that can identify genetically related viral variants. The essence of HMA is that two amplicons (reference and sample) are mixed together in approximately equal amounts and heated to 94°C - 95°C to denature them into single strands. On cooling this mixture (placing tubes in ice water immediately after denaturation), re-annealing will occur to form the following products

(i) the two original homoduplexes

(ii) two heteroduplexes composed of complementary single strands from the specimen and the reference plasmid

The products are then run through a non-denaturing Polyacrylamide gel (PAGE). Migration is influenced by the degree of base pairing of the homoduplexes and heteroduplexes. Complementary single strands (homoduplexes) will migrate more quickly than identically size amplicons that have mismatched bases. The mismatches cause 'bubbles' and retard their movement through the gel matrix. Insertions and deletions make Indels; these are "bent" structures that retard even more through the gel matrix.

1.7.3 Sequencing

Sequencing DNA is considered the gold standard procedure for HIV genotyping because it identifies not only point mutations and polymorphisms, but also insertions, deletions, and frame shift. HIV genotyping by DNA sequencing is based on a modification of the Sanger dideoxynucleotide chain termination chemistry [76]. The most common form consists of a reverse transcription (RT) reaction that converts a region of the viral RNA into cDNA followed by several nested PCRs and direct sequencing of PCR products.

Direct sequencing is one of the most accurate and automated methods for scanning DNA fragments for polymorphisms and mutations. Of several fluorescent-based approaches to sequencing PCR products, sequencing with Taq DNA polymerase and with dye-labelled dideoxy chain terminators is the most versatile because it eliminates the need for specially modified sequencing primers [77].

Different fluorescent dyes are incorporated into each of the four dideoxynucleotides, the end result is a fragment of the HIV genome labelled with four fluorescent dyes, one for each of the four nucleotides that make up the DNA molecule. The reaction products are concentrated and loaded in a single well on a polyacrylamide gel. During electrophoresis, these fluorescent-labelled products are excited by an argon laser and detected near the bottom of the gel while the sequencing information is acquired directly by computer. Although sequencing remains the most accurate approach for characterizing virus genomes, this method is time-consuming and requires a considerable investment in terms of equipment and reagents, as well as a lot of experience. This precludes the use of sequencing for monitoring the distribution of HIV strains in populations.

1.8 Viral Fitness

As a consequence of (i) rapid viral turnover [78], (ii) high rate of incorrect nucleotide substitutions during reverse transcription [79], and (iii) recombination capabilities [80-85], HIV, like other RNA viruses, exists as quasispecies, i.e. a set of distinct but related viral genomes, allowing very rapid adaptation to host immune pressure and antiretroviral drug therapy. Domingo and Holland [86] suggested that quasispecies compete amongst each other and that the most fit variant will impose itself. Hence, viral fitness is a parameter defining the replicative adaptability of an organism to its environment.

It is determined by viral factors (genome replication, protein synthesis/processing, particle assembly and release) and host factors (variety of cell types and microenvironments, anatomical compartments, ARV treatment, immune pressure). Continuous production of viral quasispecies results in adaptation to most environmental changes [86, 87].

1.8.1 Viral Fitness and Transmission

Even though the populations of HIV virus are extremely heterogeneous the quasispecies recovered from primary HIV-1 infections is more homogeneous with a narrow genetic distribution as compared to the donor HIV-1 quasispecies. The transmitted variants are likely less fit than the donor quasispecies due to the genetic bottle neck. The selective factors imposing the bottleneck are diverse and likely include: (i) host factors such as innate immune response, (ii) density of target cells at the site of infection, and (iii) number of transmitted virions.

The viral properties selected during initial infection may not be the same attributes necessary for efficient dissemination and rapid turnover during acute disease. SI/X4 HIV-1 isolates often dominate the quasispecies late in disease and yet the NSI/R5 variant is typically transmitted to a recipient [88]. Preferential transmission of NSI/R5 over SI/X4 HIV-1 isolates is contradictory to increased turnover of SI/X4 HIV-1 over NSI/R5 isolates in culture [58, 89], however in vivo findings suggest that NSI/R5 HIV-1 isolates may out-compete the SI/X4 variants at the site of primary infection, one report suggests that the NSI/R5 isolates only predominate after a temporary expansion of SI/X4 HIV-1 isolates is quenched by an activated immune response [90].

1.8.2 Viral Fitness and Diseases progression

General observations have linked HIV-1 phenotype and disease progression. For example, the faster replicating SI/X4 HIV-1 isolates are generally isolated during AIDS or in late HIV disease, whereas the slower replicating, NSI/R5 strains generally predominate during asymptomatic stages [57, 58, 89]. Another observation shown by several studies is the appearance of SI/X4 isolates does coincide with rapid decline in CD4 cells, a burst in viral load, and the onset of AIDS. However, SI/X4 isolates are inconsistently isolated in late stages of disease and are not a prerequisite for progression or AIDS [88, 91, 92].

The dogma that all SI/X4 isolates are more fit in cell culture than NSI/R5 isolates have been challenged by competing several NSI/R5 - SI/X4 pairs in PBMC cultures [93]. Although most SI/X4 were more fit in cell culture, NSI/R5 isolates from rapid progressors could out-compete SI/X4 isolates from long-term survivors or even SI/X4 isolates from patients displaying typical HIV-1 progression [93].

Besides changes in co-receptor usage/cell tropism, evidence that HIV-1 genetic alterations could affect disease progression was clearly demonstrated in a few long term non-progressor patients (LTNP) shown to harbour HIV-1 strains with *nef* deletions [94, 95]. Daniel *et al.* [96] had generated several HIV-1 clones with similar *nef* deletions, all of which were replication defective in PBMC cultures. Interestingly, several of the LTNP infected with *nef*-deleted viruses have eventually progressed to AIDS (after >10 years of asymptomatic disease) [97]. There is also evidence that HIV-1 subtype can influence disease progression, but this is very controversial. In Kenya, where subtypes A, C and D co-circulate, the plasma levels were highest and the CD4 Cell counts were lower in subtype C [98]. A study in neighbouring Uganda, where A and D are dominant, showed that D was associated with more rapid progression. A study in Sweden, however, found no difference in disease progression between A, B, C and D [99].

1.8.3 Viral Fitness and circulating HIV subtypes

At least two to three separate zoonotic jumps from chimpanzees into humans led to the disproportionate spread of HIV-1 groups M (main), O (outlier), and N (non-M/non-O) [54, 100, 101]. Several studies relate the differential spread of HIV-1 group M, group O and HIV-2 in the human population (i.e. in vivo fitness) to difference in transmission [102, 103] and pathogenesis [104-109].

HIV-1 group M subtypes and CRFs are unequally distributed across the globe, e.g. subtype B in the Americas and Europe whereas A, C and CRF02_AG are the most prevalent classes in Africa [81, 110].

The “founder effect” has played a key role in the predominance and extreme variation of HIV-1 group M over group N or O isolates e.g. subtype B epidemic in the North, the subtype C epidemic in Southern Africa, the E epidemic in South East Asia etc. Increase “fitness” has also played a role, there are areas in the world (e.g.; Central West Africa), where viruses clearly competes e.g. HIV-1 displacing HIV-2; within HIV-1: group M prevailing over O, within M some subtypes and recombinants are prevailing over others.

Over the past decade, there has been a considerable shift in the epicenter of the HIV-1 epidemics from Sub-Saharan Africa to Southern Africa, India and Southeast Asia. Subtype C has now emerged as the predominant clade in the world and accounts for at least half of all infections worldwide [111, 112]. Although subtype B likely preceded subtype C as a founder clade in India and china, most new infections in these countries are attributed to subtype C isolate or intersubtype B/C recombinant [112, 113]. This rapid insurgence of subtype C may be due to a founder effect or to intersubtype fitness difference.

1.9 Antiretroviral Therapy

Treatment of HIV-1 involves the use of antiretroviral drugs. The introduction of these drugs has resulted in reduced plasma viral loads, increasing CD4 T counts and ensuing reduced disease progression in the developed world. These drugs target specific stages of the viral life cycle. There are currently 4 major classes of antiretroviral drugs in use: fusion inhibitors, nucleoside reverse transcriptase inhibitors (N-RTIs), non-nucleoside reverse transcriptase inhibitors (NN-RTIs) and protease inhibitors (PIs)

1.9.1 Binding and fusion inhibitors

Viral entry, the first stage of the life cycle is a particularly attractive target; because it requires multiple interactions between the env proteins and the host receptors. The first step in the entry process is the binding to the cellular membrane. Binding inhibitors block the interaction between the gp120 part of the envelope and its cellular receptors CD4, CCR5, CXCR4 or DC-SIGN.

Current inhibitors include; T22, Cyanovirin-N, Pro-542 and Dextran sulfate, these target cell attachment and CD4-gp120 binding. Other inhibitors include; Tak-779, RANTES derivatives, AMD3100 and ALX40-4C, these target the co-receptor binding process.

Following binding, the viral envelope has to fuse with cellular membrane in order to infect the target cell. gp41 mediates the final part of viral entry. During co-receptor binding, conformational changes at the N terminal of gp41 results in the formation of a triple-stranded coiled coil. Further conformational changes result in the formation of a six-helix bundle, which is the direct cause of membrane fusion.

Fusion inhibitors prevent the entry process by binding to the HIV envelope protein gp41, the viral transmembrane glycoprotein that is responsible for fusion with host cells. By blocking this step fusion inhibitors interfere with the conformational change (folding) of the envelope molecule required for fusion with the target cell membrane [114]. The prototype fusion inhibitor is T-20 (Enfuvirtide, FuzeonTM) [115], is a synthetic peptide derived from a naturally occurring amino acid sequence known as heptad repeat 2 (HR2) found in gp41. As a mimic of HR2, T-20 competitively binds to a second region of gp41 heptad repeat 1 (HR1). Thus T-20 prevents interaction between HR1 and HR2 and inhibits the conformational change of gp41 that is necessary for fusion of virions to host cells.

1.9.2 Reverse transcriptase inhibitors (RTIs)

The historically firstly developed antiretrovirals targeted the reverse transcriptase enzyme and they are still the corner stone of current HAART therapy. Reverse Transcriptase is unique to retroviruses, with the exception of Hepatitis B virus and retrotransposons (mobile genetic elements) [116]. The essential role of RT in the viral life cycle and its uniqueness to these viruses makes it an ideal target for drug therapy. Drugs targeting RT belong to two classes of compounds: The Nucleoside Reverse Transcriptase Inhibitors (N-RTIs) and the Non-Nucleoside Reverse Transcriptase Inhibitors (NN-RTIs).

1.9.3 Nucleoside reverse transcriptase inhibitors (N-RTIs)

These drugs are chemically and structurally similar to nucleoside analogues, but they lack two or three of the 5' phosphate and a free 3'OH group in their sugar ring. Once they are phosphorylated by cellular enzymes, they act as competitive inhibitors of the natural nucleoside triphosphates: they are incorporated in the reverse transcript, but the absence of the 3'OH results in a premature termination of the DNA synthesis terminated [117]. Nucleoside analogues currently in use (AZT (*Retrovir*TM), d4T (*Zerit*TM), 3TC (*Epivir*TM) and its analogue FTC (*Emtriva*TM), ddI (*Videx*TM) ddC (*Hivid*TM), ABC (*Ziagen*TM)), need to be triphosphorylated within the cell to sufficiently resemble their natural counterparts and some undergo further modifications (ddI for example is converted into ddA).

Nucleotide analogues (PMPA (*Viread*TM), PMEA) resemble mono-phosphorylated nucleosides, thus avoiding the first rate-limiting phosporylation and therefore require only two additional phosphorylations to become active inhibitors of DNA synthesis [118-120]. NRTIs are good antiretrovirals, but are marred by resistance. With the emergence of NRTI mutations, RT can remove chain terminators and re-continue chain elongation [121, 122]. Thus these inhibitors can only delay viral replication, but do not stop it irreversibly. In addition, these drugs are more efficient in activated cells and not resting ones, since the former contain higher levels of nucleoside-phosphorylating enzymes [118-120].

1.9.4 Non-nucleoside reverse transcriptase inhibitors (NN-RTIs)

Non-nucleoside reverse transcriptase inhibitors (NN-RTIs) are chemically and structurally different from natural nucleosides. NNRTIs are non-competitive inhibitors and bind near the active site of the RT. Binding causes a conformational change in the active site (allosteric effect), such that it can no longer bind its nucleotides substrate; thus rendering the enzyme inactive [123].

The prototype NN-RTI is Nevirapine (*Viramune*TM). Other drugs include Efavirenz (*Sustiva*TM) and Delavirdine (*Rescriptor*TM) [124]. Several other molecules are under investigation including UC-781, a thiocarboxyanilide, and diarylpyrimidines TMC-120 (Dapivirine) and TMC125 [125-129].

NNRTIs show higher potency relative to their NRTI counterparts, however resistance still to NNRTIs occurs. Resistance is usually as a result of mutations that occur in the binding site of the NNRTI or other part of the enzyme, apart from the active site, such that the RT can no longer recognize the drug as a substrate. Mutations can also occur in the active site such that even after the conformational change induced by the NNRTI occurs, the RT active site can still recognize its substrate and hence remain functional even when the NNRTIs are active. A number of resistance-associated mutations have been documented for NNRTIs. These include: K103N mutation (Nevirapine, Efavirenz and Delavirdine) and Y181C (Nevirapine and Delavirdine).

1.9.5 Protease inhibitors (PIs)

The viral protease is required for the production of mature infectious particles after release. Due to its crucial function in viral infectivity, this enzyme is one of the most exploited for use in drug design. Protease inhibitors are competitive inhibitors of the protease substrates.

Current PI approved include Saquinavir (FortovaseTM), Amprenavir (AgeneraseTM), Atazanavir (ReyatazTM), Indinavir (CrixivanTM), Nelfinavir (ViraceptTM), Ritonavir (NovirTM) and Lopinavir + Ritonavir (KaletraTM). Resistance to PIs occurs as a result of mutations that cause a slight change in the active site of the protease resulting in reduced affinity of the protease to these inhibitors. Primary mutations cluster near the active site of the enzyme, reducing both replicative capacity and catalytic activity i.e. reduced fitness.

The accumulation of secondary mutations helps enzymes to adapt to the primary mutation leading to gradual increase in fitness. A number of resistance-associated mutations have been documented for protease inhibitors. These include: D30N mutation (Nelfinavir). N50D mutation (Amprenavir). V82A, V82S and V82T mutations (Indinavir, Ritonavir and Lopinavir). I84V mutation (Nelfinavir, Amprenavir, Indinavir, Saquinavir, Ritonavir and Lopinavir). N88D mutation (Nelfinavir). L90M mutation (Nelfinavir and Saquinavir).

1.10 Prevention of HIV infection - Microbicides

An effective HIV vaccine is still remote [130, 131]. In the mean time, effective measures that prevent HIV infection exist. The theoretical efficacy of latex condoms for preventing HIV transmission is 100% [132, 133]. Latex condoms may also prevent HIV infections by decreasing the incidence of other sexually transmitted (STDs) that may facilitate HIV transmission [5, 134]. But condoms are not used systematically enough. Women have often no power to enforce male condom use. Female condoms, on the other hand, are more cumbersome and expensive and even less accepted than male condoms. Hence the idea to develop other preventive drugs (microbicides) that can be used by women to protect themselves, even if the male partner is unwilling to use a condom.

A 'microbicide' is any substance that can substantially reduce transmission of sexually transmitted infections (STIs) when applied either in the vagina (or rectum). Like today's spermicides, a microbicide could be produced in many forms, including; gels, creams, suppositories, films, or in the form of a sponge or a vaginal ring that slowly releases the active ingredient over time. An ideal microbicide will block infection in an early stage, at least before integration of the virus in the genetic code of the host. Moreover, a combination of drugs that target two or three steps of the virus life cycle could be necessary. Microbicides are not currently available, but scientists are pursuing over 60 product leads, and approximately 20 are in or are nearing small scale clinical trials [135].

Identification of novel microbicidal compounds is one of the most rapidly expanding areas of HIV prevention research. At present there are considerable number of candidate microbicides (drugs); targeting the different stages of the HIV infection process including inhibitors of viral attachment, of binding to (co)-receptors, of fusion, of reverse transcription and integration (*figure 5*).

Microbicides from the oldest category include detergent-like agents, which can disrupt the membrane or envelope of a wide range of sexually transmitted microbes.

Some of these products (e.g. nonoxynol-9) have been evaluated in clinical trials, but they showed a relatively low activity and high toxicity, probably due to disrupting of the vaginal epithelial cell membrane and ensuing micro-ulcerations and inflammation [136-138]. Newer drugs, interfering with the early stages of HIV infection (binding, fusion, reverse transcription) in a more specific way, showed promising in vitro results, using laboratory HIV strains and artificial host cells (T cell lines).

Nucleoside Reverse Transcriptase Inhibitors (N-RTIs) and Non-Nucleoside Reverse Transcriptase Inhibitors (NN-RTIs) respectively act as competitive and non-competitive inhibitors of reverse transcriptase (RT) [139]. In principle, both classes could be used as microbicides, but the NNRTIs have an advantage in that they do not need cellular activation to be in an active antiviral state. Hence NNRTI can directly act on the RT molecules of cell-free virus and have an equal activity in proliferating or resting cells.

UC-781 an NN-RTI of the carboxy-aniline class, was the first NN-RTI considered as topical microbicides following the demonstration of its virucidal effects [125, 140], it is presently in clinical trial for this purpose [141]. Since then new second generation NN-RTIs are being developed for possible use as tropical microbicides. TMC-120 and TMC-125 are new potent NN-RTIs that belongs to the diarylpyrimidine (DAPY) analogues, importantly, they are still active on viruses, resistant to older NN-RTI: 80% of the strains with typical mutations for NN-RTI resistance remain highly sensitive to the DAPY compounds (EC_{50} below 0.1µmol/l) [142]. DAPY compounds can adopt multiple stable modes of binding to wild type and mutant HIV-1 RT [143, 144], this property could delay emergence of resistance to DAPY compounds as compared to the first generation of NN-RTI compounds.

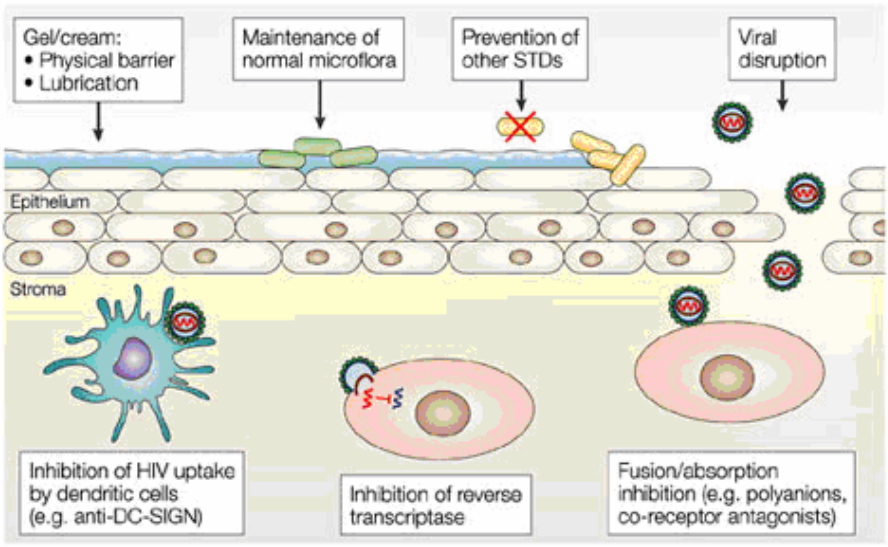

Figure 5: Potential mechanisms of action for microbicide compounds [145].

1.11 Epidemiological importance of CFR02_AG and aims of study

In the early 1990s, HIV-1 subtype analysis in different areas in Cameroon revealed the presence of diverse subtypes at different prevalence with subtype A causing almost 60% of new infections. Less then a decade later, this pattern had changed and molecular epidemiological studies showed that CRF02_AG was the most predominant circulating form of HIV-1 in Cameroon until today. Re-analysis of samples from West African location such as Senegal Equatorial Guinea, Cameroon, Nigeria, Benin and Gabon with techniques that identify a larger part of the viral genome, showed that many samples thought be to be subtype A were in fact CRF02_AG.

In a review paper by Osmanov et al, CRF02_AG was shown to have caused 5.3% new infections globally between 1998 and 2000, but 31.1% of new infections in West Africa and 6.7% in Central Africa. The sample size in this study was large (n = 4,250) and a stringent genotyping was done, sequencing two parts of the genome env + gag / pol [146].

Earlier studies with smaller numbers but in various African countries also consistently showed that CRF02_AG is clearly more prevalent than the “pure” subtype A and G in West and Central Africa [147-154]. In the mean time CRF02 viruses have now been introduced in Europe and, to a minor extent, in the US and Puerto Rico [90, 155-157].

Aims of this thesis: focus on diagnosis, fitness and prevention of CRF02_AG.

The emergence of CRF02_AG as the predominant strain causing HIV infections in West Africa may simply be a chance event. However, the fact that CRF02_AG has spread much more rapidly than other clades and other CRFs in West and West Central Africa, where many HIV-1 group M subtypes as well as group O and HIV-2 viruses co-circulate, raises concern that CRF02_AG may be favoured. Continued evolution of the molecular diversity of HIV greatly adds to the complexity of tracking the epidemic. At present, surveillance is hampered by the lack of a simple, rapid and cheap test for subtyping.

To date, no systematic large-scale attempts have been made to document the true prevalence of CRF02_AG in developing countries. This is due to the lack of cheap and rapid subtyping methods. Therefore, we aim to design a CRF02_AG specific oligonucleotide probe hybridization assay for large scale monitoring of the prevalence of CRF02_AG variants.

The predominance of CRF02_AG in the epidemic raises concern that, it may have emerged through natural selection based on a biological “fitness” advantage. In the context of HIV, fitness refers to replicative capacity in particular cellular environments, representative for either transmission (dendritic cells) or chronic pathology (CD4 T cells). It is therefore crucial to address the most relevant questions. Does CRF02 have a transmission advantage as compared to their parental strains? Does CRF02 have a replicative advantage over their parental subtypes?

In view of the alarming rate at which women acquire HIV in the developing countries, a new focus in prevention of HIV is the development of microbicides. A chemical anti-viral barrier that is applied vaginally and that women can control.

Clinical trails are likely to be carried out in Africa where prevalence of HIV is high, hence it is important to know if candidate microbicides (second generation non-nucleoside reverse transcriptase inhibitors (NNRTI)) can equally inhibit relevant circulating HIV strains, including CRF02_AG.

The aim is to study the biological importance of CRF02_AG. This study is divided into 4 parts,

(i) To Develop a probe hybridization assay for identifying Human Immunodeficiency Virus Type 1 (HIV-1) Circulating Recombinant Form (CRF) CRF02_AG

(ii) To study the viral fitness of CRF02_AG in “transmission” model and “pathogencicity” model

(iii) To study the inhibitory capacities of various reverse transcriptase inhibitors on relevant circulating HIV-1 subtypes including HIV-1 CRF02_AG: Implications for use as microbicide worldwide.

(iv) To study recently developed NNRTI’s as virucidal microbicides in a new in vitro assay, simulating the in vivo female genital tract

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2.1 Target cells

2.1.1 PHA-T-BLAST preparations

Peripheral blood mononuclear cells (PBMCs) were obtained from a HIV-1 seronegative buffy coat by Ficol Hypaque density gradient centrifugation. PBMC were stimulated in RPMI 1640 - 2mM L-glutamine (Bio-Whittaker, Verviers, Belgium) medium supplemented with 100 u/ml penicillin and 100µg/ml streptomycin (Bio-Whittaker, Verviers, Belgium) as well as 10% fetal bovine serum (FBS) (= complete medium) supplemented with 2µg/ml of PHA(phytohemagglutinin; Murex Diagnostics, UK) for 3 days and further maintained in complete medium with 1 ng/ml interleukin (IL-2) (Roche Diagnostics, Belgium). The activated PBMC were referred to as PHA-T-BLAST, since we observed that, after PHA/IL-2 stimulation, > 90 % of the cells were $CD3^+$ with blast-like morphology.

2.1.2 Monocyte derived cells (MO-DC) and $CD4^+$ T cells

Monocytes were obtained from PBMC by counter-flow elutriation and sheep erythrocyte rosetting, yielding >95% CD3- CD4+ MO and <0.5% T cells [1]. To obtain MO-DC, monocytes were cultured for 7 days in complete medium with IL4 (20ng/ml) (Roche Diagnostics, Belgium), GM-CSF (20ng/ml), (Invitrogen, Breda, The Netherlands) [2, 3]. Half of the culture medium (with cytokines) was replaced every 3 days. The MO-DC were immuno phenotyped as $CD3^-/CD4^+$, CD1a high, $CD13^+$/CD14 low, $DC\text{-}SIGN^+$ before use.

2.1.3 U87 cells

U87.CXCR4 and U87.CCR5 cell lines (National Institute for Boilogical Standards and Controls, UK.) were propagated in Dulbecco's Minimum Essential Medium (DMEM) medium supplemented with 10% fetal calf serum FCS (Bio-Whittaker, Verviers, Belgium), and 1 mg/ml G418 (Invitrogen, Breda, The Netherlands).

2.2 Plasma, sera and virus isolates

All plasma and sera samples that were tested in the probe assay study were obtained from seropositive individuals infected with HIV-1, from Bénin (West Africa), Cameroon (West Central Africa), Côte d'Ivoire (West Africa), and Belgium.

In the fitness study viruses were obtained from HIV seropositive patients attending the military hospital in Yaoundé and Douala in Cameroon. Viruses were isolated between 1996 and 1999 and none of the patients was receiving antiretroviral treatment (ART) at that time. Individual informed consent was signed by the patients.

In the microbicide study, NSI/R5, monocyte-tropic strain HIV-$1_{Ba\text{-}L}$, was provided by the National Institute of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD, USA). NSI/R5 primary isolates were obtained from seropositive African patients either consulting at the Institute of Tropical Medicine during the period 1989-1993.

2.2.1 Virus Culturing

Virus stocks were propagated and expanded in short-term cultures of PHA/IL-2 treated PBMCs.

2.2.2 Virus Titration

The 50% tissue culture infectious dose ($TCID_{50}$) was determined by titrating virus stock in PHA-T-BLAST, U87 cells and MO-DC and $CD4^+$ T cell co-cultures [4, 5].

2.3 Probe Hybridization Assay

The concept of DNA hybridization takes advantage of the ability of nucleic acids to form stable, double stranded molecules when two single strands with complementary bases are brought together under favourable conditions of temperature, pH and ionic concentration. The methodology of the probe assay involves (*figure 6*).

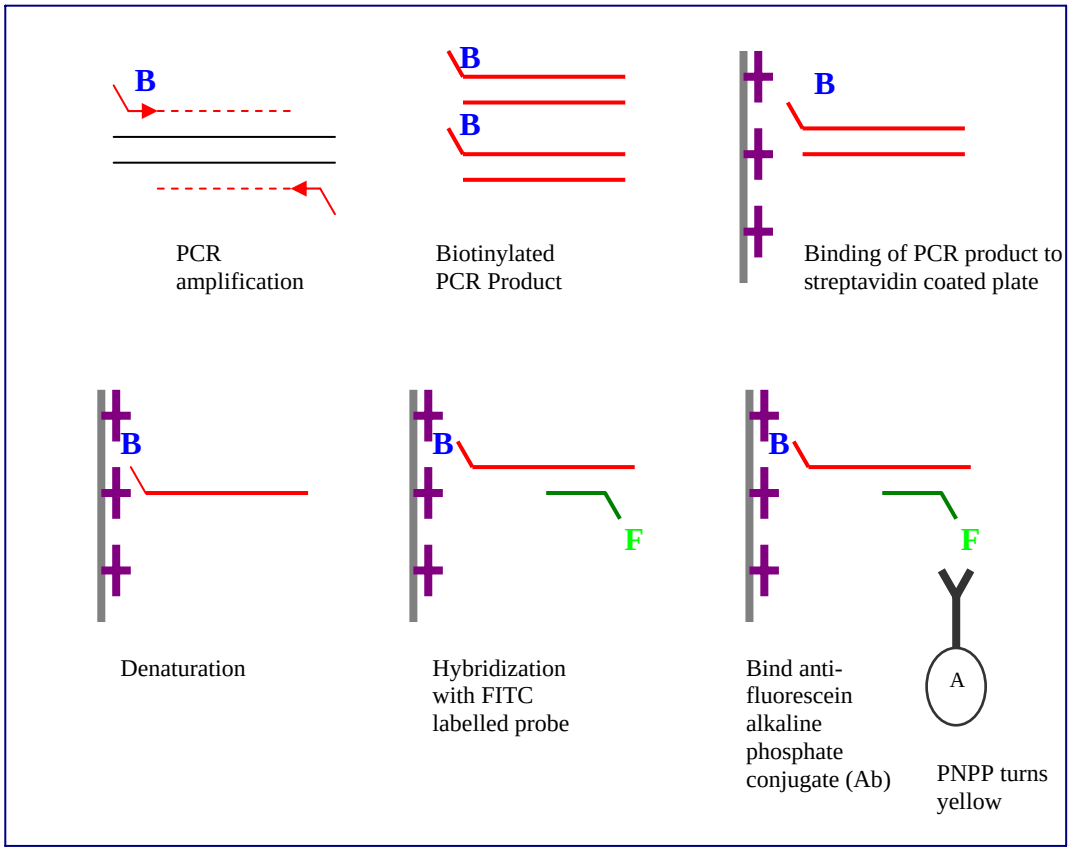
(i) A 360bp biotinylated PCR product is bound to a microtiter plate coated with streptavidin.

(ii) The bound PCR product is denatured, and the unlabelled second strand removed.

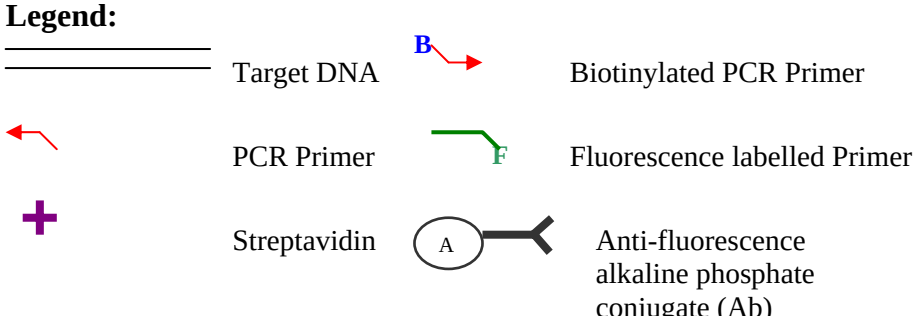
(iii) A fluorescent [Fluorescein-Iso-Thio-Cyanate (FITC)] labelled oligonucleotide probe specific for CRF02_AG is hybridized to the bound single stranded PCR product, followed by stringency washes.

(iv) The hybridized oligonucleotide probes are detected by means of anti-fluorescent alkaline phosphatase (AP) labelled antibody. The resulting complex is incubated with the colorimetric substrate para-nitrophenylphosphate (pNPP), pNPP is hydrolyzed to p-nitrophenol upon addition to complex, p-nitrophenol is yellow in colour and can be detected at 405 nm and 650nm. At 405 nm both absorbance and scattered light is measured, at 650 nm only scattered light is measured, the latter is subtracted from the former to eliminate error due to scattering.

Figure 6: Principle of the probe assay**.**



Legend:



2.3.1 Designing CRF02_AG target region and specific probes

Based on sequences for all HIV-1 group M subtypes and CRFs, available from the Los Alamos National Laboratory HIV sequence database, primers were designed to amplify a fragment of approximately 360bp covering a *gag* gene region [nucleotide positions 1063 to 1424] coding for part of p17 and p24 (according to HIV-1 HXB2 numbering Engine), where a CRF02_AG specific signature sequence was identified. Based on reported CRF02_AG sequences for the probe target region 2 distinct probes (PAg17α and PAg17β) were designed initially and evaluated on reference plasmids.

Twenty-one out of 2330 sequences in the 1999 HIV-1 database (http://hiv-web.lanl.gov/) harbouring the *gag* p17 probe target region were described as CRF02_AG. 19/21 matched the PAg17α the other 2 matched PAg17β. Evaluation and optimization of PAg17α, and PAg17β using the plasmids of the reference panel led to the design of 5 working probes. This accommodated sequence variation at the probe target regions observed in the reference plasmids (PAg17α: PAg17α1, PAg17α2, PAg17α3, PAg17α4 [HXB2 position 1161-1187], PAg17β: PAg17β1 [HXB2 position 1163-1187]).

2.3.2 Design of the Probe hybridization assay

The experimental conditions of the CRF02_AG probe hybridization assay were optimized using a panel of plasmids containing the *gag* gene of HIV-1 subtypes and CRFs. Experimental conditions were obtained whereby reference isolates could be amplified by PCR and unambiguously typed as CRF02 or non-CRF02 in the probe hybridization assay. The optimized procedure was as follows:

2.3.3 Nested PCR amplifications

Samples were amplified in 50µl with primers PGF1 and BHGHMA625. Positive control 1 (isolate DJ258) was amplified in 50µl with primers PGF1 and BHGHMA625. Positive control 2 (isolate DJ258*) was amplified in 50µl with primers F-PGF1 (F-PGF1 is the same as PGFI except of the FITC) and BHGHMA625.

2.3.4 Binding and detection

The probe hybridization assay was performed on streptavidin coated 96 well MTP (company). All wash steps were done in a volume of 230µl, and were incubated for 2 minutes at room temperature, unless mentioned otherwise. All incubations at 37°C were done in an air incubator. All incubations at 65°C were done on a hot plate (Multiblok, Labline)

2.3.5 Tris buffer based binding protocol

The MTP was equilibrated by washing three times with Tris wash buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20). One hundred micro litres of Tris binding buffer (500 mM NaCl; 10 mM Tris-HCl pH 7.5; 1mM EDTA) was added to each well. 2µl Positive control 1 (DJ258) (~100 - 250ng) was added to A1, B1, C1, D1 and C12. Two micro litres positive control 2 (*DJ258) (~100 - 250ng) was added in column 12, wells E, F, G and H (black wells in Figure 2), whereas no PCR product is added in A12, B12 and D12 (diagonally lined wells). Two micro litres of sample 1(~100 - 250ng) was added to column 2; wells A, B, C and D (dotted wells), sample 2 was added to column 3; wells A, B, C and D e.t.c. A maximum of 21 samples could be loaded per plate. The plate was incubated for 30 min at 37^0C, followed by 15 minutes at room temperature. Plate was washed twice with Tris wash buffer.

2.3.6 Denaturation and hybridization

With exception of wells G12 and H12, bound PCR product was denatured by adding 230 µl 0.15M NaOH and incubated at room temperature for 10 min. Washes with 0.15M NaOH were repeated three times with respective incubation times of 5 min, 2 min, and 2 min (in wells G12 and H12, 0.15M NaOH was substituted by Tris neutralization buffer (100 mM Tris-HCl pH 7.5) in each step), The MTP was then washed three times with Tris neutralization buffer to stop the denaturation process, followed by washing once with Hybridization buffer (0.6 M NaCl; 20mM Na_xPO_4 pH 7; 1mM EDTA; 1X Denhardts solution [1% Ficoll 400 (Sigma F-4375); 1% polyvinylpyrrolidone 360 (Sigma PVP-360); 1% BSA (Sigma B-4287)]. Three micro litres FITC-labelled probes (1 pmol / µl) were added to 100 µl hybridization buffer in each well, except in the wells B12, C12, D12, E12, F12, G12 and H12.

The MTP was incubated for 2 hours at 65^0C on the Multiblock. The hybridization/probe solution was immediately discarded after hybridization.

2.3.7 Stringent washes

The MTP was washed twice for 5 min with 6XSSC/0.1% SDS buffer at 37 ^{0}C in an air incubator, then washed twice for 30 min at 65 ^{0}C with 1.8X SSC. The wash buffer was immediately discarded after each step.

2.3.8 Tris buffer based detection

The MTP was blocked with antibody incubation buffer (100 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% (w/v) blocking reagent (Roche) for 10 minutes at room temperature. The blocking solution was then discarded. One hundred micro litres of diluted antibody (1/2500, Anti-fluorescein-AP Roche 1 426 346) was transferred in each well (1/2500 dilution). The MTP was incubated for 45 minutes at 37 ^{0}C, followed by 15 minutes at room temperature. The MTP was washed 4 times with Tris wash buffer.

Optionally the Tris wash buffer may not be discarded at the last wash, and the MTP can be stored at 4°C overnight or during the weekend until the addition of detection solution and the reading. The wash buffer was discarded, “hybridized oligonucleotide – antibody complex” bound to MTP, was incubated with 190 µl of the calorimetric substrate Para-nitrophenylphosphate (pNPP) (i.e. one Tris-buffer tablet and one pNPP tablet (Sigma) dissolved at room temperature in 20 ml sterile water) in each well. pNPP is hydrolyzed to p-nitrophenol upon addition to the complex; P-nitrophenol is yellow in colour and can be detected at 405 nm and 650nm. Absorbance readings were measured immediately using an ELISA reader over a period of 2 hours. The wells B12 and D12 were used as blanks. At 405 nm both absorbance and scattered light is measured, at 650 nm only scattered light is measured, the latter is subtracted from the former to eliminate error due to scattering. The Kincalc program automatically calculates OD/min of each sample’s reaction to the different probe(s).

2.4 Viral Fitness

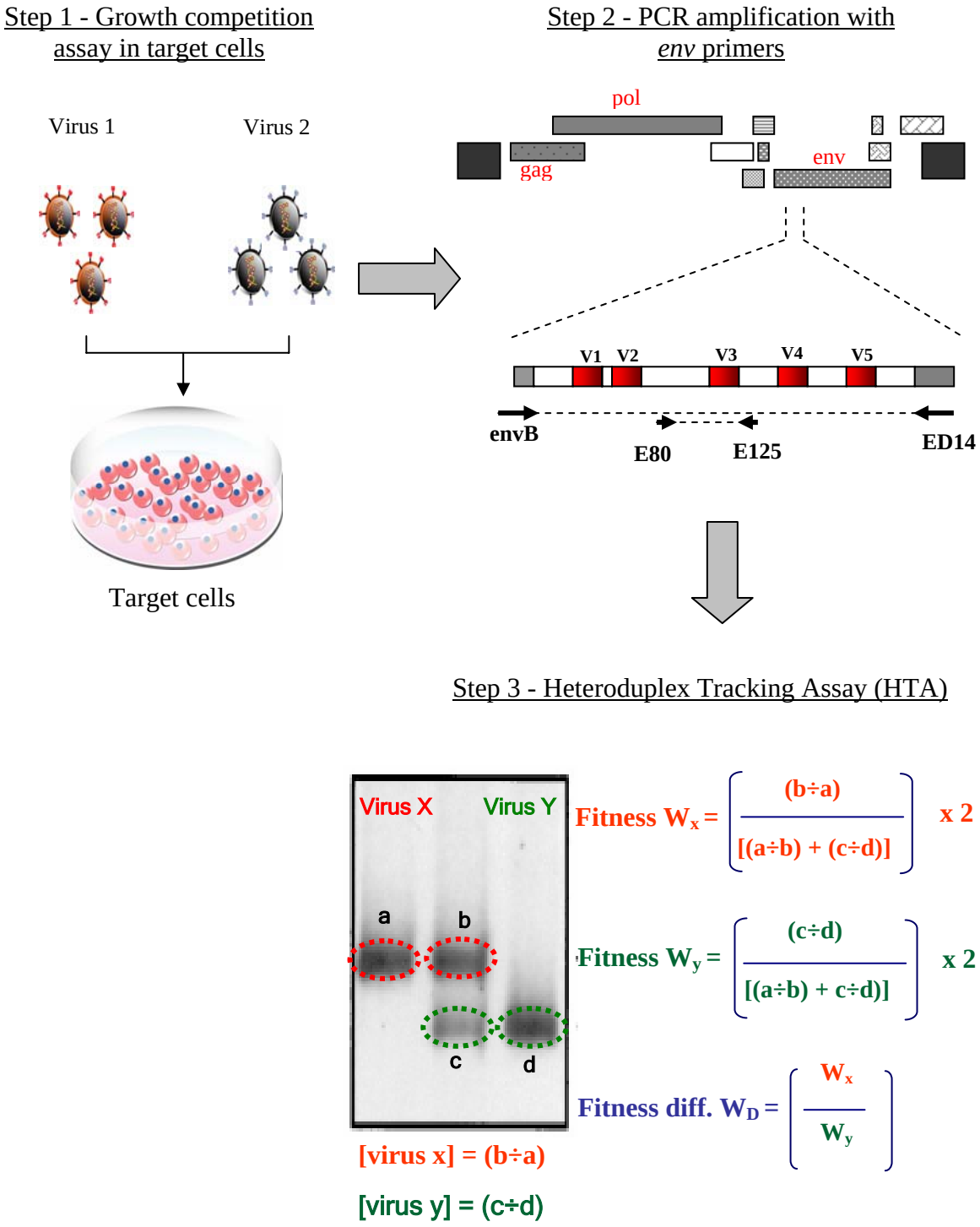
Viral fitness is a parameter defining the replicative adaptability of an organism to its environment and is best defined by assaying the replication capacity during growth competition experiments. Growth competition experiments between two viral isolates provide the internal control lacking in mono infections and relative fitness can be directly compared in dual infections, since populations in culture compete until one clone or quasispecies outgrows the other [6].

2.4.1 Dual infection/competition assays

Dual infection/competition experiments were performed as previously described [6]. Eleven CRF02_AG were competed against 5 subtype A and 5 subtype G, in PHA-T-BLAST from one donor (same donor as the $TCID_{50}$ determination and same blood draw) in 24 well culture plates and in duplicate. A second set of competitions was performed, in duplicate, using all available NSI/R5 isolates; in MO-DC from another donor ($TCID_{50}$ determination was done n U87.CD4 R5 cells). In these competition experiments, cells ($2x10^5$ PHA-T-BLAST or $1x10^6$ MO-DC) were infected with two isolates at equal multiplicity of infection ($5x10^{-4}$ MOI for PHA-T-BLASTs or 1×10^{-3} MOI for MO-DCs).

Uninfected cells were used as HIV-negative controls and mono-infected cultures of each virus were used as positive controls. The cultures were kept at 37^0C in 5% CO_2 for 24 hours and residual virus was washed away (3X) with 1X phosphate-buffered saline pH 7.4. Infected cells were re-suspended in complete medium with IL-2 for PHA-T-BLASTs or complete medium without IL-2 for MO-DCs and kept at 37^0C in 5% CO_2 for 14 days. Half the culture medium was replaced (with IL-2 for PHA-T-BLAST, without IL-2 for MO-DCs) twice a week. Cell free supernatant were collected at day 7, 10 and 14 and analyzed for p24 content using an in-house p24 ELISA assay. The cells were harvested as soon as the ELISA of cultures showed overflow. Thus, PHA-T-BLAST infections were harvested at day 10 whereas MO-DC infections were harvested at day 14. Cells of harvested cultures were kept at -80^0C for subsequent analysis (*figure 7*).

Figure 7: Principle of the growth competition assay



2.4.2 Heteroduplex Tracking Assay

Infected cells were harvested and genomic DNA extracted from lysed PHA-T-BLAST using the QIAamp DNA blood kit (Qiagen). DNA was PCR amplified using a set of external primers (envB; 5'-AGAAAGAGCAGAAGACAGTGGCAATGA-3' and ED14; 5'-TCTTGCCTGGAGCTGTTTGATGCCCCAGAC-3') and nested amplification was done using the primers E80 (5'-CCAATTCCCATACATTATTGTC-3') and E125 (5'-CAATTTCTGGGTCCCCTCCTGAGG-3') to produce a ± 480bp fragment, encoding the C2-C4 *env* region (2, 45). Both first round and second round PCR amplifications were carried out in 100µl reaction mixture under defined cycling conditions (*figure 7*) [6].

Subsequently, nested PCR products were then analyzed by Heteroduplex Tracking Assay (HTA) to estimate the amount of virus produced by each isolate in the competition, relative to the amount of virus produced in monoinfections [6]. The same genomic region of two subtype B HIV-1 strains (i.e. VI969-6 and JR-FL) was amplified and used as probes in the HTA. For this amplification the E80 primer was radiolabelled using T4 polynucleotide kinase (PNK, Gibco BRL) and 2µCi of [γ-32P] ATP. Radiolabelled PCR-amplified probes were separated on 1% agarose gel and then purified using QIAquick gel extraction kit (Qiagen). Reaction mixtures containing DNA annealing buffer (100mM NaCl, 10mm tris-HCl [pH 7.8], 2mM EDTA, 10 µl of unlabelled PCR-amplified DNA from the competition cultures and approximately 0.1 pmol of radioactive probe DNA.

For each competition two HTAs were performed using both probes. DNA amplified from competition and probe were denatured at 95°C for 3 min, 37°C for 5 min and then rapidly annealed in wet ice. After 30 minutes DNA heteroduplexes were resolved on 5% TBE non-denaturing polyacrylamide (PAGE) gel (Bio-Rad) for 1h 15min at 200V. Gels were then dried for 45 minutes at 80°C and exposed on phosphor imaging screen overnight. Films were later scanned with a phosphor imager (Cyclone, PerkinElmer) followed by analysis using the OptiQuant software package from PerkinElmer.

2.4.3 Estimation of relative viral fitness

Virus production (*ws*) of each isolate in a dual infection was calculated by dividing the amount of isolate in the dual infection and the amount of the same isolate in a monoinfection. From these *ws* values, relative fitness (*W*) values for each virus were obtained using the formula [*W = (ws1/ (ws1+ ws2))* x *2],* where *ws1* and *ws2* are virus production of isolate 1 and 2, respectively. The ratio of relative fitness (W_D) values of each HIV variant in the competition is a measure of fitness difference between both HIV strains ($W_D = W_M / W_L$), where W_M and W_L correspond to the relative fitness of the more and the less fit viruses, respectively (*figure 7*).

2.5 Pre-treatment of PLL immobilized cell free virus

An ideal microbicide should act on both cell-free and cell associated virus and able to block infection in an early stage, at least before integration of the virus in the genetic code of the host. In order to evaluate if certain drugs are able to inactivate cell-free virus, before it infects the target cells, we developed an elegant *in vitro* system to investigate the impact of an anti-retroviral compound on the cell–free virus, without interfering with cells. Pre-coating plates with positively charged poly-l-lysine enhance binding of cells on the surface on plastic plate; the negatively charged ions of the cell membrane will interact with PLL by an electrostatic force. Using the same principle we immobilized virus to plates pre-treated with PLL. We further explore the ability of the immobile virus to still infect target cells and document the antiviral effects of the new class of compounds (*figure 8*).

2.5.1 Non-Nucleoside reverse transcriptase inhibitors

Two novel NNRTIs, TMC120 and TMC125 (DAPY analogues) designed by the Centre for Molecular Design Janssen Pharmaceutica (CMD) were used to determine their antiviral activity against a number of HIV strains. UC781 (NNRTI), AZT (NRTI) and PMPA (NtRTI) were selected as the reference compounds.

2.5.2 Treatment and replication inhibition

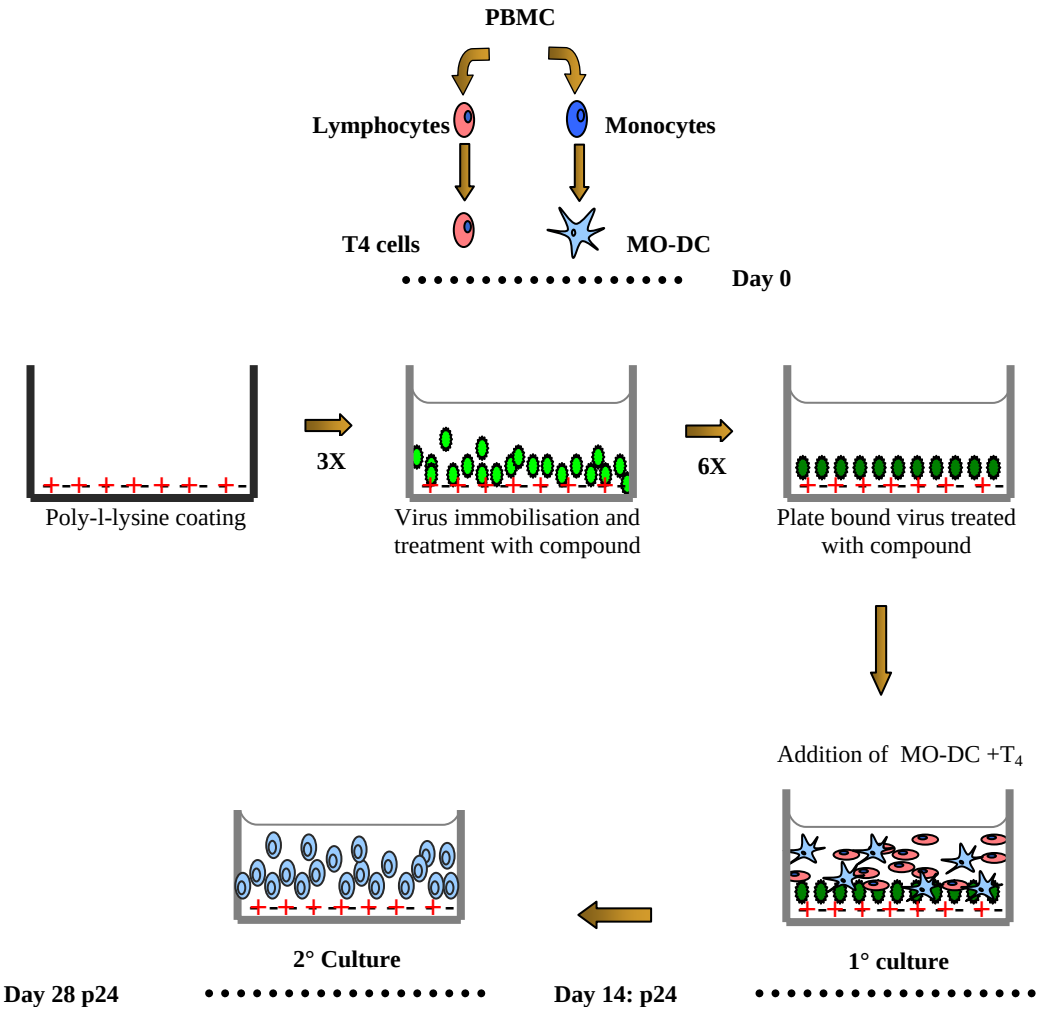
Compound stocks were prepared by dissolution in dimethyl sulfoxide (DMSO) at 10 or 100 milli Molar. Poly-l-lysine (100µg/ml) (Sigma-Aldrich, Bornem Belgium) in Phosphate Buffered Saline (PBS) (Whittaker, Verviers, Belgium) was coated on 96 well flat bottom polystyrene plates (Microtest^{TM}96, Becton Dickinson France S.A., Meylan Cedex, France) at room temperature. After 1 hour plates were washed 3 times with PBS (Bio Whittaker, Verviers, Belgium). To these plates, we added 100 µl of virus at a multiplicity of infection (MOI) of 10^{-4} and 100µl of compound, prepared in 7-fold dilution (final concentration of 100,000 nM - 0.1 nM) in complete medium i.e. RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 U/ml) and streptomycin (100µg/ml) and 10% Fetal Bovine Serum (FBS) (Biochrom KG, Berlin, Germany).

After an incubation period of 1 hour at room temperature, plates were washed thoroughly (6X) with PBS to remove excess compound and unbound virus. The plate-bound and compound-pre-treated virus was then seeded with 100µl MO-DC ($4x10^5$ cells/ml) and 100µl autologous resting CD^4+ T cells ($2x10^6$ cells/ml) in complete medium. Each compound concentration was tested in 6 fold. Half of the culture medium (without cytokines and compound) was replaced twice every week during the primary culture phase of 14 days.

2.5.3 Rescue of latent virus PHA/IL-2 stimulated PBMCs

Three days before the end of the primary culture, PBMC from a different donor were stimulated with phytohemagglutinin (PHA) at 0.5 µg/ml and IL-2 at 10 U/ml. At day 14 of the primary culture, cells were washed 3X (by centrifugation of the plates at 400g for 10 minutes) to remove cell-free virus in the supernatant. The PHA/IL-2 stimulated PBMCs (further referred to as PHA T BLAST) were washed and resuspended at 0.5 x 10^6 cells/ml in complete medium with IL-2 at 10U/ml. Two hundred µl of this suspension was added to all the wells of the DC/T4 cultures for a secondary culture of another 14 days. Half of the culture medium (including IL-2, but without compound) was replaced twice every week.

Figure 8: Poly-l-lysine based *in vitro* model to study inhibitory capacity of NN-RTIs



2.5.4 HIV antigen and EC_{50} of antiretroviral compounds determination

One hundred microlitres of day 7, day 14 (primary culture), day 21 and day 28 (secondary culture) supernatants were collected and inactivated with an equal volume of Nonidet P-40 (NP40) (Calbiochem, EMD Biosciences, Inc. Darmstadt, Germany). HIV antigen in the supernatants was measured using a modified in-house monoclonal p24 (HuMab-HIVp24, Biomaric NV, Ghent, Belgium), enzyme linked immunosorbent assay (ELISA) [7].

Optical density (OD) readings at 450nm were transformed into HIV antigen concentrations, using a standard curve of HIV-1_{Ba-L} stock dilutions, the p24 content of which was previously determined with a commercial kit (Innogenetics). HIV antigen concentration was plotted against compound concentration (Microplate Manager PC program, BIORAD Laboratories Inc, Hercules, California, USA) and regression analysis was performed on the linear part of the curve to calculate EC_{50} value (equivalent to the compound concentration required to reduce HIV antigen production by 50%).

2.5.5 Quantification of provirus HIV-1 DNA

Provirus DNA was quantified with a commercial kit according to the manufacturer's instructions (Amplicor HIV-1 Amplification kit. Roche Molecular Systems, Branchburg, NJ, USA) [8]. The cut-off value for a negative sample was 10 DNA copies / 1 X 10^6 cells.

2.6 Immune suppressive activity of compound on antigen driven-cell proliferation.

Autologous co-cultures of monocyte derived dendritic cells (MO-DC) and CD4(+) T cells were incubated with 10µg/ml of recall flu antigen (Influenza Virus Antigen A/Sydney/5/97 (H3N2) (Resvir-13) NIBSC, Potter Bars, Herts, U.K) in the presence of compound in complete medium i.e. RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 U/ml) and 2.5% Human Serum (HuS). Seven compound concentrations ranging from 100,000nM – 100nM were tested in six replicates. MO-DC + CD4(+) T cells with antigen but without compound were used as positive control.

The cultures were incubated for 6 days in an incubator (37 °C and 5% CO_2). On day six, 1 µCi of [methyl- ^{3}H]-Thymidine (Tra.120 Amerischam Pharmacia, Buckingham, UK) was added to each well and further incubated for 7 hours. Cells were then harvested on glass filter paper and incorporated radioactivity was measured in a scintillation counter (Top count; Canberra-Packard, Zellik, Belgium) and expressed as counts per minute (cpm). The immune-suppressive concentration (ISC_{50}) is defined as the compound concentration inhibiting 50% of CD4(+) T lymphocyte proliferation.

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Development, Evaluation, and Validation of an Oligonucleotide Probe Hybridization Assay To Subtype Human Immunodeficiency Virus Type 1 Circulating Recombinant Form CRF02_AG.

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Abstract

We have developed and validated an oligonucleotide probe hybridization assay for human immunodeficiency virus type 1 (HIV-1) circulating recombinant form (CRF) CRF02_AG. In the p17 coding region of the *gag* gene, a CRF02_AG-specific signature pattern was observed. Five working probes were designed to discriminate CRF02_AG infections from infections by all other documented subtypes and CRFs in an enzyme-linked immunosorbent assay-based oligonucleotide probe hybridization assay. Nucleic acids were extracted from a panel of HIV-1-positive plasma samples from Cameroon, Bénin, Côte d'Ivoire, Kenya, Zambia, and Belgium and from blood spots from The Gambia. CRF02_AG (n = 147) and non-CRF02 (n = 100) samples were analyzed to evaluate and validate the oligonucleotide probe hybridization assay. The CRF02_AG-specific oligonucleotide probe hybridization assay has a high sensitivity and specificity, with good positive and negative predictive values in regions of high and low prevalence. A validation of the assay with West and West Central African samples indicated a sensitivity of 98.4% and a specificity of 96.7%. The oligonucleotide probe hybridization assay as a diagnostic tool will allow for rapid screening for CRF02_AG. This could be used to track the HIV epidemic in terms of documenting the real prevalence of CRF02_AG strains and will complement efforts in vaccine development. Moreover, this technology can easily be applied in laboratories in developing countries.

Introduction

Genetic variation is the hallmark of retroviruses and is also apparent in human immunodeficiency virus type 1 (HIV-1). HIV-1 displays important genetic variability which is driven by the high error rate of the reverse transcriptase (23), the presence of viral RNA as a dimer, allowing recombination to occur (12), the high turnover rate of HIV-1 in vivo (24), and selective immune responses.

By genetic analyses, HIV strains collected from around the world have been shown to have substantial diversity. Representatives of different "pure" (nonrecombinant) subtypes, namely subtypes A, B, C, D, F, G, H, J, and K, and of 15 circulating recombinant forms (CRF), namely CRF01 to CRF15, were proposed based on a near-full-length genome analysis (http://hiv-web.lanl.gov/). The epidemiology of HIV-1 subtypes and CRFs is characterized by their differential distributions and varying levels of significance as driving causes of the pandemic on a regional and global basis. The largest proportion of HIV-1 infections in the year 2000 was due to subtype C strains (47.2%), followed by subtype A and CRF02_AG (27%) and subtype B strains (12.3%) (20).

Discrimination between subtype A and CRFs comprising fragments of subtype A is hampered by relatively small genetic distances between the parental subtype A virus and the respective subtype A fragments in CRFs. This often results in low-confidence classification when sequences of suboptimal length are phylogenetically (re)analyzed. In addition, it has not been possible to discriminate subtype A from CRF02_AG by *env* heteroduplex mobility assays (HMAs), and alternate experimental conditions are needed to discriminate between subtype A, CRF01_AE, and CRF02_AG by *gag* HMA (9). The prototype strain of CRF02_AG, HIV-1 IbNG, was initially reported as a new subtype A isolate from Ibadan, Nigeria (11). An analysis of the full-length sequence revealed that HIV-1 IbNG was an A/G recombinant (4). Recent and retrospective molecular epidemiology studies indicated that in West and West Central Africa, CRF02_AG infections represent 50 to 70% of the circulating strains (1, 5, 15, 16, 17, 19, 21, 27, 28).

In contrast to the high CRF02_AG prevalence in the West African and West Central African countries Cameroon, Gabon, and Equatorial Guinea, CRF02_AG infections are scarce in the Republic of Congo (26), the Democratic Republic of Congo (31), and Eastern and Southern African countries (3). Outside Africa, CRF02_AG has been introduced in Europe (8, 10, 18, 25, 29), and to a minor extent, in the United States (13).

To date, there have been few systematic large-scale attempts to characterize HIV isolates, and especially CRFs, emerging from different parts of the world. As such, our knowledge of the distribution of HIV strains in different populations and about changes in that distribution over time is rather limited. A major challenge in the design and evaluation of efficacious subtype-dependent candidate HIV-1 vaccines is the development of techniques for large-scale HIV genetic characterization to document the true prevalence rates of HIV subtypes and CRFs in developing countries. Here we describe the design and potential use of a CRF02_AG-specific oligonucleotide probe hybridization assay for large-scale monitoring of the prevalence of CRF02_AG variants.

Materials & Methods

Samples

A reference panel of 25 plasmids, containing the complete *gag* gene of HIV-1 strains belonging to group M subtypes A to H, CRF01_AE, and CRF02_AG, was available (14). An evaluation panel of plasma samples (Bénin, n = 59; Cameroon, n = 53; Kenya, n = 50; Zambia, n = 10; Belgium, n = 80) were selected based on subtype information obtained in previous studies (9, 16, 17). A validation panel of plasma samples (Côte d'Ivoire, n = 30; Cameroon, n = 60) and dried blood spot samples (The Gambia, n = 10) were obtained from HIV-1-positive individuals from whom samples were generally taken in 2000-2001. As the "gold standard," phylogenetic classification of the *gag* probe target fragment was used.

RNA extractions and RT-PCR

RNA extractions were performed as previously described (2). Reverse transcription-PCR (RT-PCR) (Access RT-PCR; Promega, Leiden, The Netherlands) was performed according to the manufacturer's recommendations (10 pmol of each primer, 10 mM dNTP mix, 25 mM $MgSO_4$). The primers and primer positions (according to the HIV-1 HXB2 numbering) (HIV Sequence Database [http://hiv-web.lanl.gov/]) were as follows: H_1GHMA101, 5′-TAGTATGGGCAAGCAGGGAG-3′ (HXB2 positions 890 to 909); and H1Gag1844, 5′-ACAGCATGCTGTCATCATTTCTTCTAGTG-3′ (HXB2 positions 1814 to 1843). The cycle protocol was 45 s at 48°C (cDNA reaction), followed by 2 min at 94°C; 40 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 90 s; and 1 cycle for 7 min at 68°C. Amplified DNA (1 μl) was subjected to a second-round PCR using primers PGF1 (5′-ATAGAKRTAAAAGACACCAARGAAGC-3′) (HXB2 positions 1063 to 1088) and BHGHMA625 (5′-B-CATTCTGCAGCTTCCTCATTGAT-3′) (HXB2 positions 1402 to 1424; biotin labeled). Cycling conditions were 2 min at 94°C and 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 60 s, followed by 1 cycle of 7 min at 72°C. Nested PCRs were carried out in a 50-μl reaction mixture containing 10 pmol of each primer, 20 mM dNTP mix, and 25 mM $MgSO_4$.

Oligonucleotide probe hybridization assay procedure

(i) Streptavidin-coated MTP binding and detection

The oligonucleotide probe hybridization assay was performed in streptavidin-coated 96-well microtiter plates (MTPs). All wash steps were done in a volume of 230 μl, with the plates being incubated for 2 min at room temperature, unless mentioned otherwise. All incubations at 37°C were done in an air incubator. All incubations at 65°C were done on a Multi-Blok Heater 2004-ICE (Lab-Line Instruments, Melrose Park, Ill.).

(ii) Tris buffer-based binding protocol

MTPs were equilibrated by washing three times with Tris wash buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20). One hundred microliters of Tris binding buffer (500 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA) was added to each well. For each sample, 2 µl of PCR product 1 (~100 to 250 ng) was added per well and per probe (combination) to be tested. Allowing for appropriate controls (see below), a maximum of 21 samples could be loaded per MTP. MTPs were incubated for 30 min at 37°C, followed by 15 min at room temperature. MTPs were then washed twice with Tris wash buffer.

(iii) Denaturation and hybridization

Bound PCR products were denatured by the addition of 230 µl of 0.15 M NaOH and incubation at room temperature for 10 min. Washes with 0.15 M NaOH were repeated three times, with incubation times of 5, 2, and 2 min. MTPs were then washed three times with Tris neutralization buffer (100 mM Tris-HCl [pH 7.5]), followed by washing once with hybridization buffer (0.6 M NaCl, 20 mM Na_xPO_4 [pH 7], 1 mM EDTA, 1× Denhardt's solution, 1% Ficoll 400 [F-4375; Sigma-Aldrich, St. Louis, Mo.], 1% polyvinylpyrrolidone 360 [Sigma-Aldrich], 1% bovine serum albumin [B-4287; Sigma-Aldrich]). Three microliters of fluorescein isothiocyanate (FITC)-labeled probes (1 pmol/µl) was added to 100 µl of hybridization buffer in each well. MTPs were incubated for 2 h at 65°C on a Multi-Blok heater. The hybridization-probe solution was immediately discarded after hybridization.

(iv) Stringent washes

MTPs were washed twice for 5 min each with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate buffer at 37°C in an air incubator and then were washed twice for 30 min each at 65°C with 1.8× SSC. The wash buffer was immediately discarded after each step.

(v) Tris buffer-based detection

MTPs were blocked with antibody incubation buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% [wt/vol] blocking reagent [1096176; Roche Diagnostics Belgium]) for 10 min at room temperature. The antibody incubation buffer was then discarded. One hundred microliters of diluted antibody (1/2,500; anti-fluorescein-AP antibody) (1426346; Roche Diagnostics Belgium) was transferred to each well. MTPs were incubated for 45 min at 37°C, followed by 15 min at room temperature. MTPs were washed four times with Tris wash buffer. The wash buffer was discarded. (Alternatively, the Tris wash buffer was not discarded at the last wash, and the MTPs were stored at 4°C overnight.)

The hybridized oligonucleotide-antibody complex bound to the MTP was incubated with 190 μl of the colorimetric substrate *para*-nitrophenylphosphate (pNPP) (i.e., one Tris buffer tablet [T-8790; Sigma] and one pNPP tablet [N-2770; Sigma] dissolved at room temperature in 20 ml of sterile water) in each well. pNPP is hydrolyzed to *p*-nitrophenol upon addition to the complex, and *p*-nitrophenol is yellow and can be detected at 405 nm. At 405 nm, both absorbance and scattered light is measured, and at 650 nm, only scattered light is measured, so the latter was subtracted from the former to eliminate errors due to scattering. Absorbance readings were measured immediately by using an enzyme-linked immunosorbent assay reader, whereby the kinetics were monitored every 5 min over a period of 2 h. The Kincalc program was used to automatically calculate the optical density (OD) of each sample's reaction to the probe(s).

(vi) Controls on each MTP

As positive and negative controls for probe reactivity, the probe target fragments of CRF02_AG and non-CRF02 samples, respectively, were processed as indicated above. For each sample, the reactivity of the PCR product with FITC-labeled PCR primer F-PGF1 was monitored as an indication of the quality and quantity of the PCR product. Wells which contained the probe and no PCR product, only a PCR product and no probe, or no PCR product and no probe were scored as negative.

As a control for the binding of PCR products to the MTPs, FITC-labeled PCR products and no probe were added and NaOH treatment was omitted, which resulted in a high-level signal. As a control for denaturation, a FITC-labeled PCR product and no probe were added, which resulted in no signal.

Data analysis

Cutoff values (CO) were determined from the 5th percentile of the OD distribution of true positives. The sensitivity was defined by the following equation: sensitivity = number of true positives/(number of true positives + number of false negatives), with true positives being CRF02_AG samples that reacted positively with one or more of the probes (OD > CO). The specificity was defined by the following equation: specificity = number of true negatives/(number of true negatives + number of false positives), with true negatives being non-CRF02_AG samples that did not react with any of the probes (OD < CO). The equation positive predictive value = number of true positives/(number of true positives + number of false positives) indicates the likelihood that a positive test result actually means that a CRF02_AG infection was identified. The equation negative predictive value = number of true negatives/(number of true negatives + number of false negatives) indicates the likelihood that a negative test result actually means that a non-CRF02_AG infection was identified.

Nucleotide sequence accession numbers

The newly obtained nucleotide sequence data were deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the following accession numbers: AJ606488 to AJ606676.

Results

Identification of a CRF02_AG-specific probe target region

Based on sequences that are representative of all HIV-1 group M subtypes and CRFs, available from the Los Alamos National Laboratory HIV sequence database (http://hiv-web.lanl.gov/), a near-full-length genome alignment was generated and screened for CRF02_AG-specific signature patterns. A candidate probe target was identified in the HIV-1 *gag* p17 coding region (Fig. 1).

Twenty-one of 2,330 sequences in the 1999 HIV-1 database (http://hiv-web.lanl.gov/) harboring the *gag* p17 probe target were described as CRF02_AG. Of these, 19 and 2, respectively, matched two different types of probe target (PAg17α and PAg17β), which were representative of two distinguished CRF02_AG subclades. Primers were designed to amplify a 360-bp probe target region by nested PCR. Five working probes were defined as follows (position numbers are according to HIV-1 strain HXB2 numbering [http://hiv-web.lanl.gov/]):

PAg17α1, 5′-CAGGAAGCAGCAGCCAAAATTACCC-3′;

PAg17α2, 5′-CAGGAAGCAGCAGTCAAAATTACCC-3′;

PAg17α3, 5′-CAGGAAGCAGTAGCCAAAATTACCC-3′;

PAg17α4, 5′-CAGGAAGCGGCAGCCAAAATTACCC-3′ (positions 1161 to 1187);

PAg17β, 5′-GCACAGGCTGCAGCCAAAATTACCC-3′ (positions 1163 to 1187).

Optimization of the oligonucleotide probe hybridization assay with reference plasmids.

For all samples of the reference panel, positive PCR products were obtained. With all five probes and the experimental conditions described above, all CRF02_AG reference plasmids scored positive and all non-CRF02_AG reference plasmids scored negative. To maximize the number of samples that could be analyzed per plate, we used probe combinations PAg17α1 plus PAg17α2 and PAg17α3 plus PAg17α4, which were evaluated to be as sensitive as the separate probes (data not shown).

Evaluation of the CRF02_AG-specific oligonucleotide probe hybridization assay.

Plasma samples were selected from countries with a high CRF02_AG prevalence (Bénin and Cameroon) and from countries with a low CRF02_AG prevalence (Belgium, Kenya, and Zambia). The efficiency of PCR amplification for each cohort was as follows: Cameroon, 98% (52 of 53 samples); Zambia, 90% (9 of 10 samples); Benin, 89% (51 of 57 samples); Belgium, 85% (68 of 80 samples); and Kenya, 36% (18 of 50 samples).

For evaluation of the CRF02_AG oligonucleotide probe hybridization assay, a panel of PCR-positive CRF02_AG (*n* = 85) and non-CRF02_AG (*n* = 71; 25 subtype A, 8 subtype B, 9 subtype C, 11 subtype D, 2 subtype F, 9 subtype G, 4 subtype H, 1 CRF01_AE, and 2 CRF06_cpx) samples were used (Table 1). As the gold standard, phylogenetic classification of the *gag* probe target fragment was used (30).

Comparisons of the phylogenetic classification of the panel samples with the probe assay results (OD values) allowed calculations of means, medians, and 5th and 95th percentiles. From these, we determined cutoff values for positivity and negativity. A sample was considered to be CRF02_AG when it reacted with an OD of >15.0 with either a probe or probe combination. A sample was considered to be non-CRF02_AG when all probes and probe combinations reacted with OD values of <15.0. The OD distribution for the evaluation panel samples is depicted in Fig. 2. The overall sensitivity of the oligonucleotide probe hybridization assay evaluation panel was 97.6% (83 of 85 samples). A 100% specificity result was documented based on the fact that none of the 71 non-CRF02 (subtypes A to H, CRF01_AE, and CRF06_cpx) samples reacted with the CRF02_AG-specific probes. Overall, 63.5% (54 of 85) of the CRF02_AG samples reacted with both PAg17α probe combinations; 12.9% (11 of 85) only reacted with probe combination PAg17α3 plus PAg17α4; 1.2% only reacted with probe combination PAg17α1 plus PAg17α2. For West African countries, probe reactivity was only observed with probe combinations PAg17α1 plus PAg17α2 and/or PAg17α3 plus PAg17α4.

In Cameroon, 61.7% (29 of 47) of the CRF02_AG samples reacted with PAg17α probes and 27.6% (13 of 47) of the samples reacted with probe PAg17β. For three Cameroonian CRF02_AG samples, reactivities to both probe PAg17α combinations and to PAg17β were observed. Two Cameroonian CRF02_AG samples did not react with any of the probes.

Table 1: Summary of the probe assay results for the evaluation panel

Genetic subtype classification	No. of sample in cohort					Probe assay result
	Belgium	**Benin**	**Cameroon**	**Kenya**	**Zambia**	
CRF02	1	37	47			82/85
A	14	8	1	2		0/25
B	8					0/8
C	3				6	0/9
D	9			2		0/11
F	2					0/2
G	3	4	2			0/9
H	4					0/4
CRF01	1					0/1
CRF06		2				0/2
Total	**45**	**51**	**50**	**4**	**6**	**81/156**

Table 2: Summary of the probe assay results for the validation panel

Genetic subtype classification	No. of sample in cohort			Probe assay result
	Cameroon	**Côte d'Ivoire**	**The Gambia**	
CRF02	31	26	5	61/62
A	16	1		1/17
B				0/0
C				0/0
D	2		1	0/3
F	2			0/2
G	2			0/2
H	3			0/3
CRF01	1			0/1
CRF06			1	0/1
Total	**57**	**27**	**7**	**62/91**

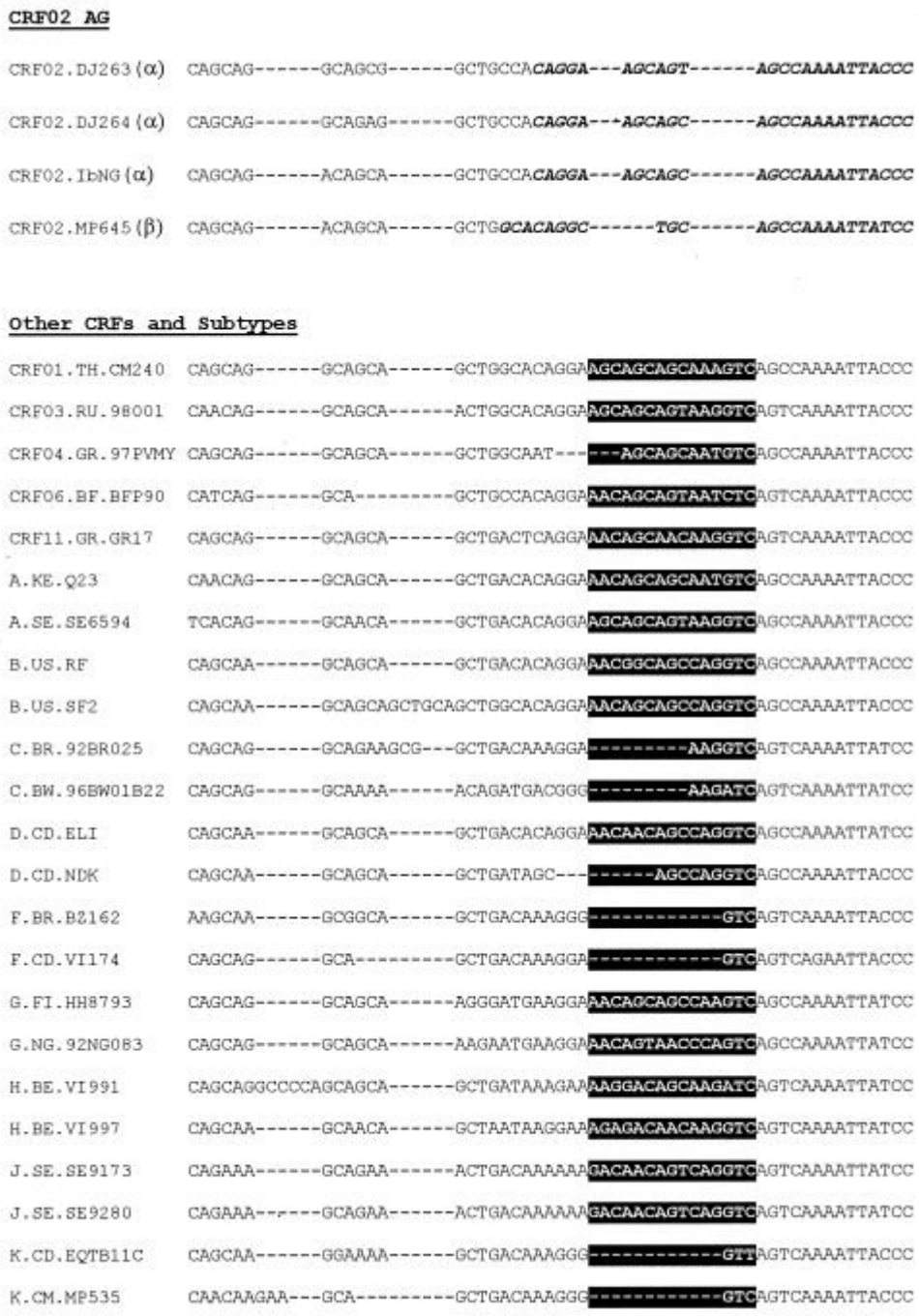
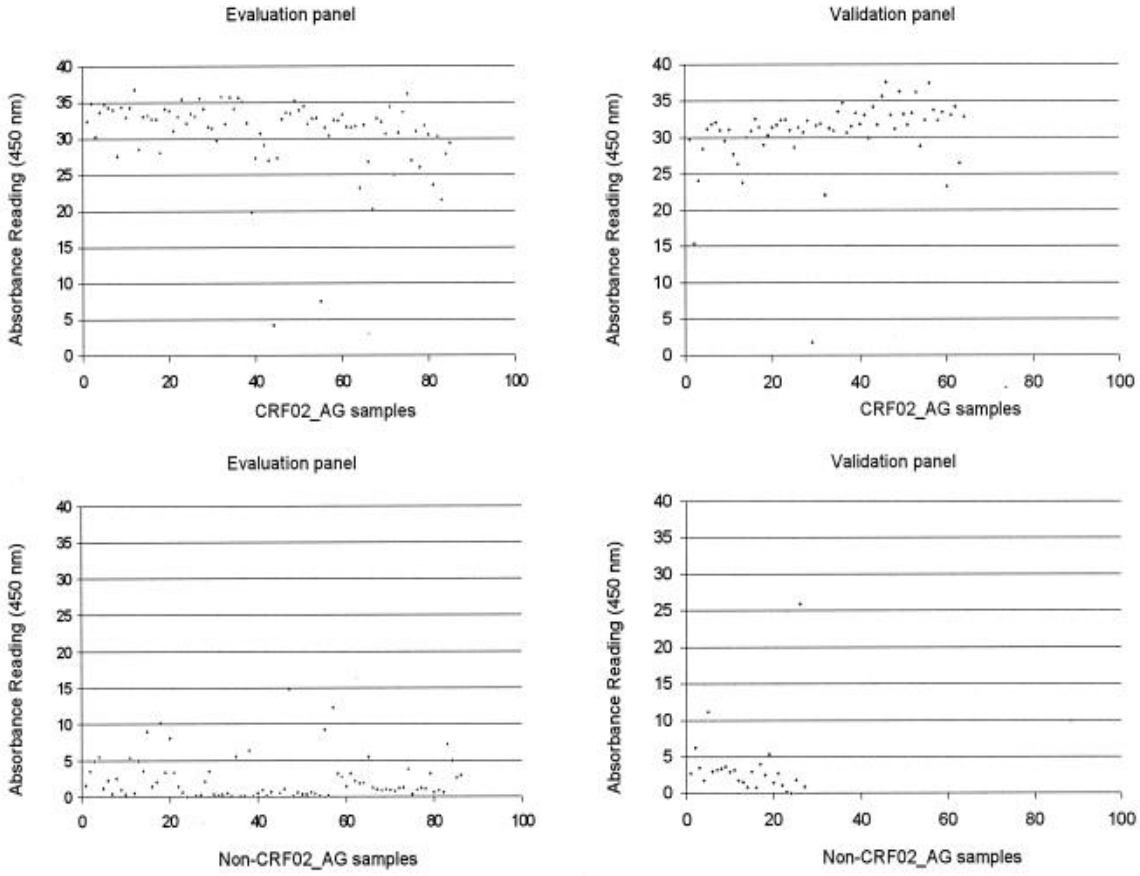


Figure 1: Alignment of the conserved signature specific for CRF02_AG with all other subtypes and CRFs comprising fragments of subtype A. Black boxes indicate amino acid insertions and deletions within different subtypes and CRFs compared to probes (in bold and italics) specific for CRF02_AG.

Validation of the CRF02_AG-specific oligonucleotide probe hybridization assay. Samples from Côte d'Ivoire (*n* = 30), Cameroon (*n* = 60), and The Gambia (*n* = 10) had been sent under code for validation of the oligonucleotide probe assay. The efficiency of PCR amplification for each cohort was as follows: Côte d'Ivoire, 90% (27 of 30 samples); Cameroon, 95% (57 of 60 samples); and The Gambia, 80% (8 of 10 samples).

For the oligonucleotide probe hybridization assay, CRF02_AG predictions for the validation panel of the different cohorts were as follows: Côte d'Ivoire, 92.6% (25 of 27 samples); Cameroon, 57.9% (33 of 57 samples); and The Gambia, 71.4% (5 of 7 samples). Subsequently, the code was revealed and the assay results were compared with the subtype classifications of the validation panel that were obtained by HMAs and/or sequencing results for *gag* and/or *env* gene fragments (Table 2). In cases of discrepant results, the probe target fragment was sequenced and analyzed. All but one CRF02_AG sample from Côte d'Ivoire were classified correctly as CRF02_AG infections by the oligonucleotide probe hybridization assay, although the CRF02_AG-specific probe target of the false-negative sample was conserved. No false-positive samples were documented.

The samples from The Gambia were correctly identified as CRF02_AG or non-CRF02_AG. For the Cameroonian cohort, one false-positive sample was detected. Of 32 CRF02_AG samples, 9 (28.1%) were reactive with the PAg17β probe. For one Cameroonian CRF02_AG sample, reactivities to both probe PAg17α combinations and to PAg17β were observed. The OD distribution for the validation panel samples is depicted in Fig. 2. The overall sensitivity for the validation panel was 98.4% (62 of 63 samples), the specificity was 96.3%, the positive predictive value was 98.4%, and the negative predictive value was 96.3%.



Figue 2: OD distribution of evaluation panel samples. Oligonucleotide probe hybridization assay results are depicted according to genetic subtype classifications of CRF02_AG and non-CRF02_AG samples. A sample was considered to be CRF02_AG if it reacted with an OD value of >15.0 with either a probe or probe combination. Only the highest OD value obtained with probe combination PAg17α1 plus PAg17α2 or PAg17α3 plus PAg17α4 or with probe PAg17β is indicated.

Discussion

The aim of this study was to develop an oligonucleotide probe hybridization assay for the identification of HIV-1 CRF02_AG infections. This assay was configured to distinguish CRF02_AG from all other subtypes and CRFs. The close genetic distance between subtype A fragments in CRF02_AG and subtype A strains hampered differentiation between CRF02_AG and subtype A by *env* HMAs (7) as well as by a one-tube real-time isothermal amplification subtyping method described by de Baar et al. (6).

A DNA enzyme immunoassay genotyping method for the *env* gene developed by Plantier et al. (22) showed a sensitivity of 86.6% (13 of 15 samples) for identifying CRF02_AG infections. The newly developed oligonucleotide probe hybridization assay showed high signal reactions to probes, differentiating CRF02_AG from other subtypes (A to H) and CRFs (CRF01 and CRF06). The oligonucleotide probe hybridization assay was validated and had a sensitivity of 98.4%, a specificity of 96.7%, a positive predictive value of 98.4%, and a negative predictive value of 96.7%, which make the assay very reliable. The sensitivity of PCR amplification of the probe target region depended on the cohort studied.

Four working probes (PAg17α1 to -α4) were sufficient to identify all CRF02_AG isolates in the West African cohort; in contrast, all five probes (PAg17α1 to -α4 and PAg17β1) were needed to identify CRF02_AG isolates in the West Central African cohort. This may indicate a further evolution of CRF02_AG in Cameroon or a founder effect by one CRF02_AG variant in West Africa.

The value of the probe assay for determining better estimates of the prevalence of CRF02_AG will complement efforts in vaccine development and evaluation. This assay will be a rapid and economical tool for use in the large-scale screening of this particular subtype at vaccine trial sites, especially in West Africa. The high sensitivity and specificity of the test also imply that there will be high positive and negative predictive values in regions of high and low prevalence.

A common drawback of using probes for genotyping in the context of HIV-1 is the huge diversity of viruses in place and time. The creation of subtype-specific probes that allow for the identification of a particular subtype by a difference of one point mutation at the probe target region has been evaluated for subtype C, with a disappointing sensitivity and specificity (results not shown). The CRF02_AG probes were designed based on a distinct signature whereby a deletion pattern compared to other subtypes and CRFs is conserved.

We realize the shortcomings of the probe assay in terms of being representative of the results for the complete genome. There is a need for continuous evaluation of the probe assay, since as HIV diversity increases with time, new variants will arise that may require adaptation of the CRF02_AG probes that are used. Therefore, the aging of epidemics and cocirculation of other subtype strains may influence, through mutation and recombination, the representativeness of the probe assay result.

The actual future role of the CRFs in the global pandemic must be monitored. The probe assay as a diagnostic tool will allow rapid screening for CRF02_AG. This could be used for tracking of the HIV epidemic in terms of documenting the real prevalence of CRF02_AG virus infections. This finding may have implications on future vaccine, diagnostic, and treatment strategies, because with the extensive movement of people between continents, the chance of recombinants becoming epidemic outside of Africa increases.

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Higher Replicative Capacity of Human Immunodeficiency Virus Type 1 (HIV-1) Circulating Recombinant Form (CRF02_AG) Over its Parental Subtypes Could Explain the Predominance of CRF02_AG in West and West Central Africa

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Abstract

The aim of this study was to test the hypothesis that, the predominance of CRF02_AG recombinant in West Central Africa is related to a higher replicative fitness. Primary isolates (11 CRF02_AG, 5 subtype A and 5 subtype G) were obtained from a well-described Cameroonian cohort. Dual competition assays and mono-infections were carried out at equal multiplicities of infection in activated T cells and monocyte derived dendritic cells (MO-DCs) in parallel. Competitions were harvested as soon as the p24 ELISA showed abundant viral production. Genomic DNA was extracted and PCR amplified for the C2-C4 env region of the integrated provirus. Heteroduplex tracking analysis (HTA) was then performed on the PCR amplicons. Based on the relative amount of provirus of each isolate in the dual infection versus the mono-infection, the relative replicate fitness (W) was calculated.

In activated T-cells, direct dual infection/competition experiments clearly indicated that CRF02_AG isolates had a significant replication advantage over the "pure" subtype A and subtype G, and the higher fitness of CRF02_AG was evident for isolates from both AIDS and non-AIDS patients and was not dependent of biophenotype of the viruses (X4 or R5). In MO-DC cultures, CRF02_AG isolates showed a slightly higher but not significant replicative advantage.

In conclusion, we observed a higher ex vivo replicative capacity of CRF02_AG isolates, independent of the disease stage and bio-phenotype but dependent on cellular environment. This fitness advantage may contribute to a dominant spread of CRF02_AG over A and G subtypes in West and West Central Africa.

Introduction

Mutation and recombination are important mechanisms by which HIV evades immune and drug pressure (48). Recombinant strains of HIV-1 have been observed worldwide (12, 15, 26, 32, 51, 60). To date there are 18 (eighteen) Circulating Recombinant Forms (CRFs) of which at least three, CRF01_AE, CRF02_AG and CRF03_AB are of major epidemiological importance. CRF01_AE (6, 15, 37,) and CRF02_AG (7, 18, 24) are causing heterosexual epidemics in Asia and West and West Central Africa, respectively. Circulation of CRF03_AB (25, 27) in Russia, appears to have been accelerated by intravenous injection of a locally produced opiate contaminated with HIV infected blood.

In a review paper by Osmanov et al, CRF02_AG was shown to have caused 5.3% new infections globally between 1998 and 2000, but 31.1% of new infections in West Africa and 6.7% in Central Africa. The sample size in this study was large (n = 4,250) and a stringent genotyping was done, sequencing two parts of the genome env + gag / pol. (41). Earlier studies with smaller numbers but in various African countries also consistently showed that CRF02_AG is clearly more prevalent than the "pure" subtype A and G in West and Central Africa. (1, 8, 13, 21, 23, 31, 33, 36-38, 43, 54). In the mean time CRF02 viruses have now been introduced in Europe and, to a minor extent, in the US and Puerto Rico (10, 22, 34, 40).

The emergence of CRF02_AG as the predominant strain causing HIV infections in West Africa may simply be a chance event. However, the fact that CRF02_AG has spread much more rapidly than other clades (including clade A and G) and other CRFs in West and West Central Africa, where many HIV-1 group M subtypes as well as group O and HIV-2 viruses co-circulate, raises concern that CRF02_AG may be favored, in terms of superior replication fitness and/or transmission efficiency.

Several studies relate the differential spread of HIV-1 group M, group O and HIV-2 in the human population (i.e. in vivo fitness) to differences in transmission (9, 17) and pathogenesis (16, 19-20, 28, 30, 59). Rapid disease progression may lead to higher viraemia at a younger age, when individuals are more sexually active hence leading to increased transmission. Recent findings on the in vitro replicative fitness of diverse human immunodeficiency viruses support the hypothesis that the relative replicative fitness and the prevalence of viral types and subtypes are directly related. It was shown indeed that HIV-1 group O and HIV-2 primary isolates had reduced replicative in activated T cells and in dendritic cells as compared to HIV-1 group M primary isolates of subtypes A, B, C, D and CRF01_AE, corroborating with the much higher prevalence of group M, as compared to group O and HIV-2 (2).

In the present study, we wanted to test the hypothesis that the predominance of CRF02_AG recombinant in West Central Africa is related to a higher replicative fitness. Therefore, we performed pair-wise competitions of a number of primary CRF02 strains with primary "pure" A and G strains, all sampled in the same area of Cameroon. In order to mimic two important micro-environments, we performed the competitions in activated T cells and dendritic cells (DC). Primary activated T cells can easily be generated by mitogen stimulation of peripheral blood mononuclear cells (PBMC). Primary DCs are more difficult to obtain, but monocyte-derived dendritic cells (MO-DC) can be generated abundantly. They combine an interstitial-like phenotype with expression of DC-SIGN, a relative preference for productive infection with NSI/R5 HIV and a high T cell stimulatory capacity. All these characteristics of MO-DC make them a good model of genital mucosa DC, which are thought to have a crucial role in the early events during transmission (39, 56-58).

Materials and Methods

Generation of target cells

Peripheral blood mononuclear cells (PBMC) were obtained from a HIV-1 seronegative buffy coat by Ficol Hypaque density gradient centrifugation. PBMC were stimulated with 2µg/ml of phytohemagglutinin (PHA) for 3 days and further maintained in RPMI 1640 - 2mM L-glutamine medium supplemented with 10% fetal bovine serum (FBS), 1 ng/ml interleukin (IL-2), 100 u/ml penicillin and 100µg/ml streptomycin. This medium will further be referred to as "complete medium with IL-2"; the activated PBMC will be referred to as PHA-T-BLAST, since we observed that, after PHA/IL-2 stimulation, > 90 % of the cells were CD3 +.

Monocytes were obtained from PBMC by counter-flow elutriation and sheep erythrocyte rosetting, yielding >95% CD3- CD4+ MO and <0.5% T cells (58). To obtain MO-DC, monocytes were cultured for 7 days in RPMI 1640 supplemented with 10% FBS, IL4 (20ng/ml), GM-CSF (20ng/ml), 100u/ml penicillin and 100µg streptomycin (49, 52). Half of the culture medium (with cytokines) was replaced every 3 days. The MO-DC were immuno phenotyped as CD3-/CD4+, CD1a high, CD13+/CD14 low, DC-SIGN+ before use.

HIV strains

Twenty-one viruses were obtained from HIV seropositive patients attending the military hospital in Yaounde and Douala in Cameroon. Viruses were isolated between 1996 and 1999 and none of the patients was receiving antiretroviral treatment (ART) at that time. Individual informed consent was signed by the patients. The choice of these 21 strains from a much larger cohort (23) was based on availability of PBMC and plasma, simultaneously obtained of these particular patients and permanently frozen at -80°C. CD4+ cell count had all been determined on fresh blood, but plasma viral load was measured for the purpose of this study, using "inhouse" real time PCR (50) (Table 1).

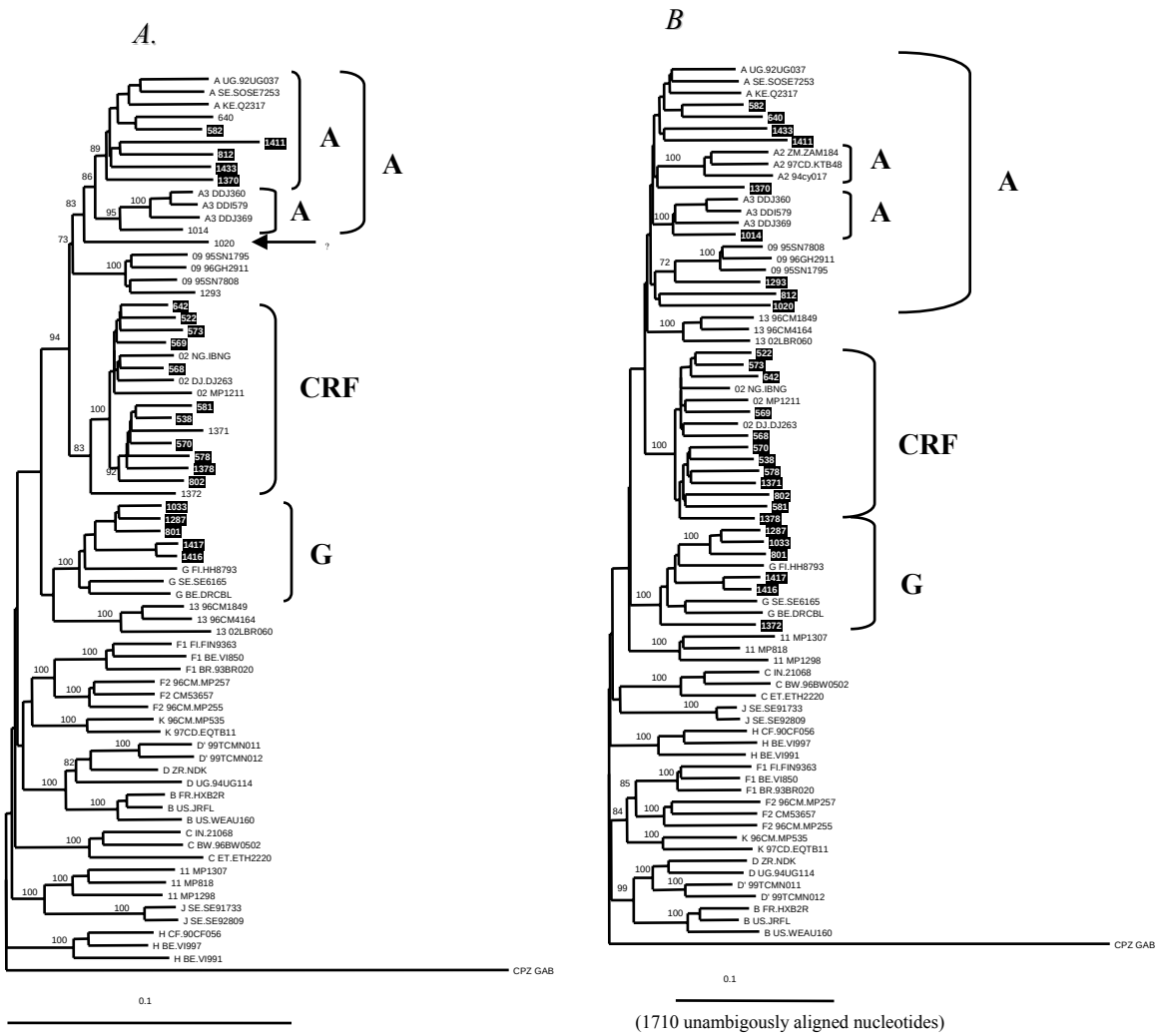
In fact, our original selection encompassed 27 isolates (11 CRFs, 10 As and 6 Gs), but 6 were discarded after full env and pol sequence (see below), because they showed unique recombination characteristics, i.e. they were not pure A, nor G, nor CRF02. Virus stocks were propagated and expanded in short-term cultures of PHA/IL-2 treated PBMC. The 50% tissue culture infectious dose ($TCID_{50}$) was determined by serial dilution of the virus stock and infecting PHA-BLAST and U87.CD4 cells expressing either CCR5 or CXCR4 (42, 47) (Table 1).

Sequencing and phylogenetic analysis

The HIV-1 strains characterized in this study were amplified from cultured patient peripheral blood mononuclear cells. DNA was extracted using the Qiagen DNA isolation kit (Qiagen S.A., Courtabeauf, France). Complete sequences for the *pol* and the *env* genes were obtained by amplifying large fragments. The first fragment, spanning the *gag-pol* region, was amplified with G00 (5'-GACTAGCGGAGGCTAGAAG-3', position 761-780 on HxB2) and HPOL4538 (5'-TACTGCCCCTTCACCTTTCCA-3', position 4994-4973 on HxB2) as outer primers. One second round fragment was obtained from a semi-nested PCR reaction with G25reverse (5'-GCAAGTGTTTTGGCTGAAGCAAT-3', position 1872-1895 on HxB2) and HPOL4538. The second fragment, covering the accessory genes region to the end of *nef* gene, was amplified with HPOL4235 (5'-CCCTACAATCCCCAAAGTCAAGG-3', position 4668-4691 on HxB2) and LSIGI (5'-TCAAGGCAAGCTTTATTGAGGCTTAAGCAG-3', positions 9647-9617/542-512 on HxB2). One second round fragment was then generated with envB (5'-AGAAAGAGCAGAAGACAGTGGCAATGA-3', position 6216-6243 on HxB2) and envM (5'-TAGCCCTTCCAGTCCCCCCTTTTCTTTTA-3', position 9116-9087 on HxB2). The two rounds of PCR used the Taq Expand Long Template PCR system (Roche, Indianapolis, USA), according to manufacturer's instructions. Cycling conditions were as follow: a denaturation step of 3 minutes at 92°C, 16 cycles of 92°C for 20 seconds, 50°C for 30 seconds and 68°C for 4 minutes, then 16 cycles with 20 seconds-increments at the elongation step, a final extension of 10 minutes and a rapid decrease of temperature to 4°C.

The amplified fragments were purified using a QiaQuik gel extraction kit (QIAGEN, QIAGEN S.A., France), and then directly sequenced with primers encompassing the *pol* and the *env* regions by using Big-Dye Chemistry (Applied Biosystems, France) according to the instructions of the manufacturer. Electrophoresis and data collection were done on an Applied Biosystems 3100 Genetic Analyzer. The electrophoregram plots were visualized and processed under DNASTAR to generate consensus from the different overlapping sequences.

The newly determined sequences were aligned with known representatives of the different subtypes, sub-subtypes and CRFs described in Africa. Sites with any gap between the sequences and areas of uncertain alignment were excluded from the analysis. Pairwise evolutionary distances were estimated with Kimura's two parameters method. Phylogenetic trees were constructed by NJ method, and the reliability of the tree topology was assessed by bootstrap analysis. Simplot 3.2 beta software (Stuart Ray, http://www.med.jhu.edu/deptmed/sray/), was used to investigate the recombinant structure of the newly sequenced genes. Similarity and bootscan plots were performed as already described. Briefly, similarity plots determined the percent similarity between a newly determined sequence and selected groups of references, by moving a window of 400 base pairs with 20 base pairs increments along the genome alignment. Similarity values were plotted at the midpoint of the 400 base pairs fragment. For the bootstrap plots, the SimPlot software performed bootscanning on neighbor joining trees by using SEQBOOT, DNADIST (with Kimura two parameters method and F84 model of maximum likelihood method, transition/transversion ratio = 2.0), NEIGHBOR and CONSENSE from the PHYLIP package for a 400 base pairs window moved along the alignment in increments of 20 base pairs. One thousand bootstraps replicates were evaluated for each phylogeny. The bootstrap values for the studied sequences were plotted at the midpoint of each window. In these two sets of analyses, the new sequences were compared with consensus sequences (50% threshold) representing the different HIV-1 clades from the same alignment used for phylogenetic tree analysis.



(1710 unambigously aligned nucleotides)

Figure 1: Phylogenetic trees in *pol* (A) and *env* (B). Sequences were generated by amplifying the full env and full pol coding regions of each isolate. Subsequently, NJ-trees were constructed and tree topology was assessed by bootstrap analysis. The SIVscpGAB sequence was used to root the tree. Eleven isolates were found to group with the CRF02_AG reference strains, five were subtype A and five were subtype G.

Dual infection/competition assays

Dual infection/competition experiments were performed as previously described (2-3, 45, 55). In short all 11 CRF02_AG were competed against 5 subtype A and 5 subtype G, (Table 1) in PHA-T-BLAST from one donor (same donor as the $TCID_{50}$ determination and same blood draw) in 24 well culture plates and in duplicate. A second set of competitions was performed, in duplicate, using all available NSI/R5 isolates, in MO-DC from another donor ($TCID_{50}$ determination was done n U87 CD4 R5 cells). In these competition experiments, cells ($2x10^5$ PHA-T-BLAST or $1x10^6$ MO-DC) were infected with two isolates at equal multiplicity of infection ($5x10^{-4}$ MOI for PHA-T-BLASTs or 1×10^{-3} MOI for MO-DCs). Uninfected cells were used as HIV-negative controls and mono-infected cultures of each virus were used as positive controls. The cultures were kept at 37°C in 5% CO_2 for 24 hours and residual virus was washed away (3X) with 1X phosphate-buffered saline pH 7.4. Infected cells were re-suspended in complete medium with IL-2 for PHA-T-BLASTs or complete medium without IL-2 for MO-DCs and kept at 37°C in 5% CO_2 for 14 days. Half the culture medium was replaced (with IL-2 for PHA-T-BLAST, without IL-2 for MO-DCs) twice a week. Cell free supernatant were collected at day 7, 10 and 14 and analyzed for p24 content using an in-house p24 ELISA assay (5). The cells were harvested as soon as the ELISA of cultures showed overflow. Thus, PHA-T-BLAST infections were harvested at day 10 whereas MO-DC infections were harvested at day 14. Cells of harvested cultures were kept at -80°C for subsequent analysis.

Subsequently, nested PCR products were then analyzed by Heteroduplex Tracking Assay (HTA) to estimate the amount of virus produced by each isolate in the competition, relative to the amount of virus produced in monoinfections (2-3, 45, 55). The same genomic region of two subtype B HIV-1 strains (i.e. VI969-6 and JR-FL) was amplified and used as probes in the HTA. For this amplification the E80 primer was radiolabelled using T4 polynucleotide kinase (PNK, Gibco BRL) and 2μCi of [γ-32P] ATP. Radiolabelled PCR-amplified probes were separated on 1% agarose gel and then purified using QIAquick gel extraction kit (Qiagen).

Reaction mixtures containing DNA annealing buffer (100mM NaCl, 10mm tris-HCl [pH 7.8], 2mM EDTA, 10 µl of unlabelled PCR-amplified DNA from the competition cultures and approximately 0.1 pmol of radioactive probe DNA. For each competition two HTAs were performed using both probes. DNA amplified from competition and probe were denatured at 95°C for 3 min, 37°C for 5 min and then rapidly annealed in wet ice. After 30 minutes DNA heteroduplexes were resolved on 5% TBE non-denaturing polyacrylamide (PAGE) gel (Bio-Rad) for 1h 15min at 200V. Gels were then dried for 45 minutes at 80°C and exposed on phosphor imaging screen overnight. Films were later scanned with a phosphor imager (Cyclone, PerkinElmer) followed by analysis using the OptiQuant software package from PerkinElmer.

Estimation of relative viral fitness

Virus production (*ws*) of each isolate in a dual infection was calculated by dividing the amount of isolate in the dual infection and the amount of the same isolate in a monoinfection. From these *ws* values, relative fitness (*W*) values for each virus were obtained using the formula [$W = (ws1/ (ws1+ ws2))$ x *2],* where *ws1* and *ws2* are virus production of isolate 1 and 2, respectively. The ratio of relative fitness (W_D) values of each HIV variant in the competition is a measure of fitness difference between both HIV strains ($W_D = W_M / W_L$), where W_M and W_L correspond to the relative fitness of the more and the less fit viruses, respectively (2-3, 45, 55)

Statistical Analysis

Mann-Whitney U tests were used to determine significant difference in viral load and CD4 counts between CRF02_AG isolates and non- CRF02_AG isolates. Competition data for each group (relative fitness values – W) was represented in median and interquartiles. Because the relative fitness values obtained for CRFs depends on the relative fitness values of the non-CRFs, one cannot directly compare fitness values of CRFs with non-CRFS. One sample t-test was used to determine if the fitness values for one group were significantly different from the value 1.0. Spearman rank test was used for correlation between fitness in MO-DC and T cell BLASTS. For all analyses, the level of significance was set at $P \leq 0.05$.

Virus	Subtype (env & pol)	Date of isolation	CD+ cells (cells/µl)	Viral Load (Log10 RNA copies/ml)	Co-receptor
MP522	CRF02_AG	15.11.96	2	5.10	X4
MP538	CRF02_AG	12.05.96	350	4.49	R5
MP568	CRF02_AG	24.02.97	266	4.91	R5
MP569	CRF02_AG	13.02.97	1029	2.95	R5
MP570	CRF02_AG	17.02.97	213	5.41	R5
MP573	CRF02_AG	30.01.97	277	5.67	R5
MP578	CRF02_AG	30.01.97	8	5.41	X4/ R5
MP581	CRF02_AG	30.11.97	8	5.80	X4
MP642	CRF02_AG	01.04.97	104	5.71	R5
MP802	CRF02_AG	15.12.97	56	5.77	X4/ R5
MP1378	CRF02_AG	12.08.99	0	5.89	R5
MP582	A	03.02.97	521	3.78	R5
MP812	A	18.12.97	310	4.52	X4
MP1370	A	12.08.99	477	4.31	R5
MP1411	A	18.08.99	105	5.18	R5
MP1433	A	16.09.99	321	4.93	R5
MP801	G	26.12.97	731	3.85	R5
MP1033	G	25.05.98	394	2.39	R5
MP1287	G	15.02.99	91	5.83	R5
MP1416	G	16.08.99	368	5.59	R5
MP1417	G	16.08.99	23	5.38	X4/ R5

Table 1: Virus and patient characteristics of twenty-one primary HIV-1 isolates obtained from Cameroon between 1996 and 1999. Complete pol and complete env were sequenced for genetic subtyping. CD4+ cell counts and viral load were determined at the time of virus isolation. Co-receptor tropism was tested on U87.CD4 cells expressing either CCR5 or CXCR4.

Results

Characterization of patient-derived HIV-1 isolates

Twenty-one HIV-1 isolates were obtained from a patient cohort in Cameroon. Fifteen isolates were found to use only CCR5, while three viruses could use only CXCR4 and three others were able to use both CCR5 and CXCR4 as entry co-receptor (Table 1). Sequencing the complete env and pol regions for genetic subtyping and subsequent phylogenetic analysis revealed that eleven isolates were CRF02_AG, five were subtype A and five were subtype G (Table 1). CD4 T counts in this patient group showed a wide variation: from 0 to > 1000/µl blood. Nine patients were in the AIDS stage of disease (CD4+ cells <200), while the remaining isolates (n=12) were obtained from less affected individuals (CD4+ cells >200) (Table 1).

Viral load determined for each patient at the time of virus isolation were generally high (> 4 logs) except in the three patients with > 500 CD4 T cells/µl. In concordance with recent observations by Fischetti et al. (14), we observed an overall trend to higher viral load in individuals infected with CRF02_AG, compared to those infected with A and G (5.53 Log10 RNA copies/mL verus 5.21 Log10 RNA copies/mL, respectively) but the difference was not significant (P-value = 0.114). Furthermore, CRF02_AG-infected individuals appeared to have reduced CD4+ cell counts compared to subjects infected with non-CRFs (210 CD4+ cell/µL versus 333 CD4+ cells/µL, respectively), but again, the difference was not significant (P-value = 0.291) (Table 1).

The replicative fitness of CRF02_AG in activated human T-cells.

Eleven CRF02_AG were competed in duplicate against five subtype A and five subtype G isolates and duplicate monoinfections of each isolates were ran in parallel. CRF02_AG isolates won 78/110 competitions (70.9%) with a median relative fitness value (W) of 1.50 (p25 = 0.96, p75 = 1.82). Subtype A isolates showed a median fitness value of 0.50 (p25 = 0.18, p75 = 1.03), and subtype G isolates had a median fitness value of 0.66 (p25 = 0.22, p75 = 1.13). Median fitness values for CRF02_AG viruses were significantly higher than those of pure subtypes A and G (P<0.001) (Fig. 2).

In previous studies (4, 11, 45, 55) it was observed that HIV-1 replicative fitness correlates directly with viral load and inversely with CD4+ cell count. Since, in our study population, CD4 counts and viral load tended to differ between CRF and non-CRF patients, we separately considered competitions of viral isolates obtained from CRF02 patients with CD4+ cell counts >200 and CD4+ cell counts <200 against the same set of A and G viruses, irrespective of the CD4 counts of the latter. In the first group (CD4<200), CRF02_AG isolates won 45/60 competitions (75.0%), with a median fitness value (W) of 1.52 (p25 = 1.07, p75 = 1.83). In the second group (CD4>200), CRF02_AG isolates won 34/50 (68.0%) of the competitions with a median fitness value (W) of 1.40 (p25 = 0.84, p75 = 1.79) (Fig. 2).

It is understood that the viral biological phenotype, determined by the co-receptor tropism, might influence HIV-1 replication in T cells and that SI/X4 viruses tend to be more "virulent" (44). Since our cohort consisted of both X4/SI and R5/NSI using isolates, we re-analyzed the data correcting for virus phenotype. X4/SI CRF won 8/8 (100%) competitions against X4/SI As and Gs. Similarly X4 CRFs won 25/32 (78.1%) competitions against R5/NSI As and Gs. However, also R5/NSI CRF won 11/14 (78.6%) competitions against X4/SI As and Gs and R5/NSI CRFs won 35/56 (62.5%) competitions against R5/NSI As and Gs. When the fitness difference (W_D) for each group of isolates was calculated, we found that the CRF02_AG isolates were on average six times more fit than viruses of subtypes A and G (P<0.001).

The replicative fitness of CRF02_AG in dendritic cells.

Since mucosal dendritic cells are thought to play an important part in the first phase of sexual transmission (29, 35, 53, 61), replicative capacity of CRF02_AG in a suitable model of DC, such as the monocyte-derived DC, will shed more light to the possible "transmission advantage" of these isolates in the pandemic. NSI/R5 viruses are consistently found early after transmission, we restricted our analysis to isolates of this phenotype (4 CRF02_AG, 1 subtype A and 3 subtype G isolates). In MO-DC, CRF02_AG isolates won 10/16 competitions (62.5%) and showed a median fitness value (W) of 1.48 (p25 = 0.68, p75 = 1.55).

Non-CRF isolates showed a median fitness value of 0.52 (p25 = 0.45, p75 = 1.18). CRF02_AGs showed a slightly higher but not significant replicative capacity as compared to the non-CRFs (P=0.229) (Figure 3).

When comparing fitness data obtained in T-cells and DCs, it was obvious most of the CRF02_AG isolates that out competed subtype A and G viruses in DCs also won the competitions in T cells. CRF02 isolates won 11/16 competitions (68.8%) in T-cells and showed a median fitness value (W) of 1.39 (p25 = 0.95, p75 = 1.62). Non-CRF isolates showed a median fitness value of 0.67 (p25 = 0.42, p75 = 1.06). In T-cells the same CRF02 isolates showed a significantly higher replicative capacity as compared to pure A and G (P=0.024) (Figure 3). CRF02_AG isolates were on average 1.5 times more fit than viruses of subtypes A and G (p = 0.201) in DCs whereas in T cells the same CRF02 isolates were 6 fold more fit than viruses of subtypes A and G ($p<0.001$).

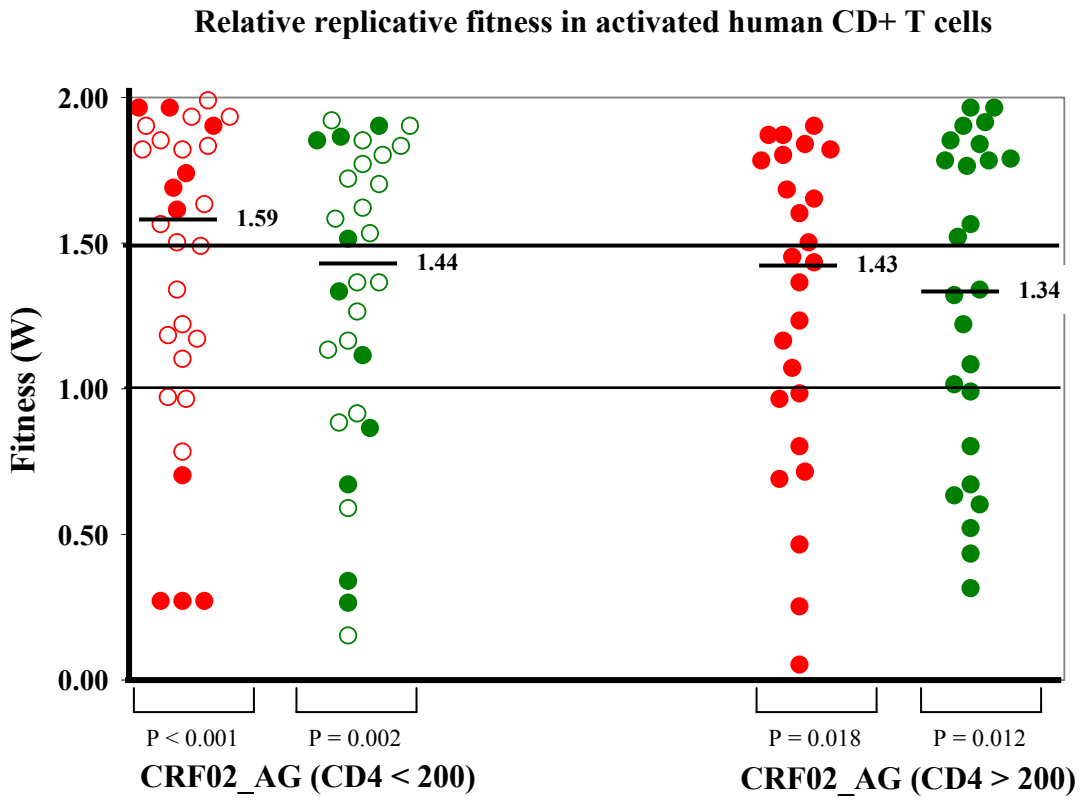


Figure 2: Relative replicative fitness in CD+ T cells: Dot plot represents 110 growth competitions in PHA/IL-2 BLAST using 11 CRF02_AG, 5 A's and 5 G's. CRF02_AG isolates won 78/110 competitions (70.9%) and showed a median relative fitness value (W) of 1.50 (represented by the thick line). Red circles indicate competitions of an X4/SI CRF02_AG against an A virus and solid red dots indicate competitions of an R5/NSI CRF02_AG against an A virus. Green circles represent X4/SI CRF02_AG competing against a G virus and solid green dots represent R5/NSI CRF02_AG competing against an G virus. The relative fitness (*W*) values for each virus were obtained using the formula [*W = (ws1/ (ws1+ ws2))* x *2],* where *ws1* and *ws2* are virus production of isolate 1 and 2 respectively. Limits of detection are a maximum relative fitness value of 2.0 and a minimum of 0.

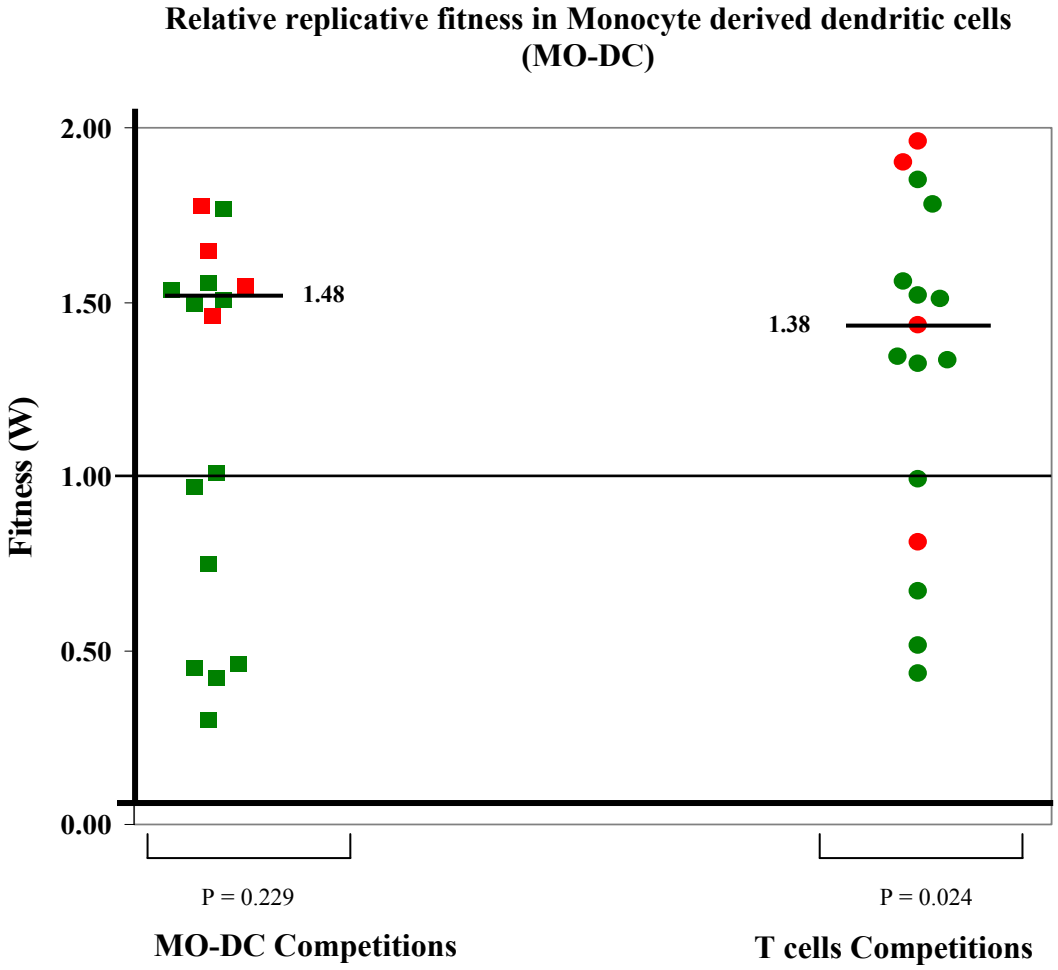


Figure 3: Relative replicative fitness in Monocyte derived dendritic cells (MO-DC): Dot plot represents 16 growth competitions in MO-DC and 16 growth competitions T cells using the same NSI/R5 viruses isolates (4 CRF02_AG, 1 subtype A and 3 subtype G isolates). Solid red square indicate competitions of a CRF02_AG competing against an A virus in MO-DC and solid green square indicate competitions CRF02_AG competing against a G virus in MO-DC. Solid red dot indicate competitions of a CRF02_AG competing against an A virus in T cells and solid green dot indicate competitions CRF02_AG competing against a G virus in T cell. In MO-DC, CRF02_AG isolates won 10/16 competitions (62.5%) and showed a median fitness value (W) of 1.48. In T cells CRF02 isolates won 11/16 competitions (68.8%) in T cells and showed a median fitness value (W) of 1.39.

Discussion

The aim of this study was to compare the replicative fitness of CRF02_AG to its pure subtypes in two cell types; activated CD4+ T cells and monocyte derived dendritic cells (MO-DCs). Dendritic cells are regarded primary targets during sexual HIV transmission and are thought to transfer the virus to T cells in the mucosa and at more distal sites such as lymph nodes. Once infection with HIV is established, activated CD4+ T cells are the preferred cell population for HIV replication. Therefore, we performed competitions in PHA-activated T-cells as a model for HIV fitness during disease ('pathogenic fitness'), while MO-DCs were used to model HIV replicative fitness upon transmission.

The dual infection assay used is based on head on competitions between two viruses of equal numbers of infectious particles. The assay provides the internal control lacking in monoinfections assays, and can detect minor differences in fitness (46).

Direct dual infection/competition experiments in activated T-cells clearly indicated that CRF02_AG isolates had a significant (on average six-fold) replication advantage over the "pure" subtype A, and subtype G patient HIV-1 isolates. These findings suggest that patients infected with the CRF02_AG strains could have a higher viremia because of a better replicating virus than those infected with non-CRF02_AG isolates. This was indeed found by Fischetti et al, who showed that asymptomatic patients infected with the CRF02_AG strains had a significantly higher viral load than those infected with non-CRF02_AG strains (14). In our own patient's sample, randomly selected from a larger cohort, the same trend of higher viremia (although non-significant) was observed.

Furthermore, this data might suggest that individuals infected with CRF02_AG strains would show a different disease progression as compared to those infected with non-CRF02_AG. However a 4 year prospective multicenter study (23) indicated that predominance of CRF02_AG strain in western and west-central Africa apparently had no major clinical consequences.

The major limitation of this study, was that date of infection was not known and subtype characterization was done on part of the env gene only, which most probably underestimates the recombinants. Therefore, in the present study we performed a more extensive sequencing and indeed, we had to discard 6/16 isolates (37.5%), classified as pure A or G with the partial env sequencing.

The higher replicative advantage of CRF02_AG over subtype A and G was also demonstrated in our "transmission model" (MO-DC cultures). In this model a 1.5 fold higher fitness of CRF versus non-CRF was observed, but even this slight advantage indicates that CRF02_AG isolates infect / replicate better in dendritic cells, potentially increasing their chances of successful sexual transmission and transport into the lymphoid tissues where the virus proceeds to infect the rest of the immune system. It is conceivable that the factors that determine fitness advantage in MO-DC cell systems are different from T-cell system and that only a slight fitness in transmission efficiency (fitness in MO-DC) may account for significant changes in virus prevalence at population level.

Recombination has proven to be an important strategy of retroviral adaptation and our observations suggest that recombination could generate HIV variants with increased replication capacity and transmission efficiency. The CRF02_AG genome is a mosaic of subtype A (gag, vpr and parts of pol, env and nef) and subtype G (LTR, rev, tat and parts of pol, env, and nef). It would be interesting to determine which part of the CRF genome is the determining factor for the increased transmission fitness. One way to investigate this question is to create artificial recombinants, by selective exchange of "pure" subtype genes with their CRF homomlogue and competing each of these artificial "chimeric" recombinants against the paternal virus.

In conclusion, we showed that CRF02_AG primary HIV-1 isolates from Cameroon are more fit in activated human T-cells and in dendritic cells compared to subtype A and G isolates from the same geographic region. These results may explain the advantageous spreading of CRF02_AG variants in West and West Central Africa during the past decade.

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Pre-incubation of cell-free HIV-1 group M isolates with non-nucleoside reverse transcriptase inhibitors blocks subsequent viral replication in co-cultures of dendritic cells and T cells.

Diarylpyrimidine (DAPY) compounds as possible microbicides.

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Abstract

In order to study the inhibitory effect of various reverse transcriptase inhibitors (RTI) on cell-free HIV, we adapted a recently described *in vitro* system, based on co-cultures of dendritic cells and resting CD4 T cells, modeling early target cells during sexual transmission. The compounds tested included the second-generation non-nucleoside RTI (NN-RTI) TMC-120 (R147681, dapivirine) and TMC-125 (R165335, travertine) as well as the reference nucleoside RTI AZT (zidovudine), the nucleotide analogue PMPA (tenofovir) and the non-nucleoside RTI UC-781. The virus strains included the reference strain HIV-1_{Ba-L} and six primary isolates, representative of the HIV-1 group M pandemic. They all display the non-syncytium-inducing and CCR5 receptor using (NSI/R5) phenotype, important in transmission. Cell-free virus was immobilized on a poly-l-lysine (PLL) treated microwell plate and incubated with compound for 1 hour. Afterwards, the compound was thoroughly washed away; target cells were added and cultured for 2 weeks, followed by an extended culture with highly susceptible mitogen-activated T cells. Viral production in the cultures was measured on supernatant with HIV antigen ELISA. Negative results were confirmed by showing absence of proviral DNA in the cells.

TMC-120 and TMC-125 inhibited replication of HIV-1_{Ba-L} with an average EC_{50} value of 38nM and 117 nM respectively, whereas the EC_{50} of UC-781 was 517 nM. Complete suppression of virus and provirus was observed at compound concentrations of 100, 300 and 1000 nM respectively. Inhibition of all primary isolates followed the same pattern as HIV-1_{Ba-L}. In contrast, pre-treating the virus with the Nt-RTI PMPA and AZT failed to inhibit infection even at a concentration of 100,000 nM. Clearly, these data suggest that NN-RTIs inactivate RT enzymatic activity of different viral clades (predominant in the epidemic) and might be proposed for further testing as a sterilizing microbicide worldwide.

Introduction

Women are at a greater risk of acquiring Human Immunodeficiency Virus (HIV) than men [1] and they account for nearly 50% of all people living with HIV worldwide [2] (UNAIDS report 2004). In some parts of Africa, the incidence of HIV is alarmingly high in younger women (age 15-25). Even though effective preventive measures (condoms) exist they are often not a feasible option for women due the need of consent from male partners. In view of this situation, a new focus in prevention is on the development of microbicides that, when applied topically, should substantially reduce transmission of HIV. An ideal microbicide should be able to block infection in an early stage, at least before integration of the virus in the host cell DNA.

Nucleoside Reverse Transcriptase Inhibitors (N-RTIs) and Non-Nucleoside Reverse Transcriptase Inhibitors (NN-RTIs) respectively act as competitive and non-competitive inhibitors of reverse transcriptase (RT) [3], but the latter have an advantage for microbicide development since NN-RTIs do not need cellular activation to be effective against HIV. Hence these compounds can directly act on the RT molecules of cell-free virus and have an equal activity in proliferating or resting cells. UC-781, an NN-RTI of the carboxyaniline class, was the first NN-RTI considered as topical microbicides following the demonstration of its virucidal effects [4-5], it is presently in clinical trial for development into a microbicide [6].

TMC-120 and TMC-125 are new potent NN-RTIs that belong to the diarylpyrimidine (DAPY) analogues. Importantly, they are active on HIV strains resistant to older NN-RTI: it was shown that 80% of the strains with typical mutations for NN-RTI resistance remain highly sensitive to the DAPY compounds (EC_{50} below 0.1µmol/l) [7]. DAPY compounds can adopt multiple stable modes of binding to wild type and mutant HIV-1 RT [8-9], this property could delay emergence of resistance to DAPY compounds as compared to the first generation of NN-RTI compounds.

Although most authors agree that mucosal dendritic cells (DC) have some important role in the initial stage of heterosexual transmission, there is disagreement as to the type of DC involved. Intra-epithelial Langerhans cells seem ideally positioned to capture HIV. Nevertheless elegant animal studies point to interstitial DC as more important targets. This might be related to the high expression of DC-SIGN, a molecule that binds gp120 and protects HIV from degradation [10-13]. For this conceptual and also for practical reasons, we decided to build our in vitro model on monocyte-derived DC (MO-DC), which resemble more closely interstitial DC rather than Langerhans cells. In previous papers, we and other showed that incubation of MO-DC with cell-free (or cell-associated) NSI/R5 HIV and co-culture with resting autologous CD4 T cells results in a productive infection, without the need for added cytokines. Moreover, we extensively demonstrated that various N-RTI and NN-RTI, added to the cells before, during and/or after infection could block HIV production [14-16].

In order to evaluate if certain compounds are able to inactivate the cell-free virus before it infects the target cells, we pre-coated plates with positively charged poly-l-lysine (PLL), enhancing binding of the negatively charged virus envelope to the surface of the plastic plate. We further explored the ability of the immobile virus to still infect target cells and documented the antiviral effects of the DAPY analogues, in comparison to the reference compounds UC-781, Zidovudine (AZT) and Tenofovir [9-(*R*)-2-(phosphonomethoxypropyl)adenine] (PMPA).

Materials and Methods

Generation of monocyte derived cells and $CD4^+$ T cells

Monocytes and lymphocytes were purified from buffy coats. The former were cultured with granulocyte-monocyte colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4) to induce DC differentiation. CD4 T cells were purified from the cryopreserved lymphocytes. The protocol was based on Sallusto et al [18, 20], with modifications previously described [15].

HIV strains, virus titration

A first series of experiments was carried out with the NSI/R5, monotropic strain HIV-1_{Ba-L}, kindly provided by the National Institute of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD, USA). Six NSI/R5 primary isolates obtained from seropositive African patients consulting at the Institute of Tropical Medicine during the period 1989-1993 were used in this study (Table 1). Subtype information was obtained by *gag*/*env* sequencing and these included HIV-1 group M subtypes A, A/G, B, C and CRF02_AG which represent the relevant circulating subtypes of the HIV pandemic. The 50% tissue culture infectious dose ($TCID_{50}$) was determined by titrating virus stock in MO-DC and $CD4^+$ T cell co-cultures [21-22].

Table 1: Panel of six primary isolates, origin, subtype and patient information

Virus code	gag/env [a]	Origin [b]
VI 820	A/A	Democratic Republic of Congo
VI 191	G/A	Belgium
CI 22	B/B	Côte d'Ivoire
VI 882	C/C	Belgium
CI 20	CRF02	Côte d'Ivoire
CA 18	CRF02	Cameroon

[a] Subtype of virus was determined by sequencing the *gag* and *env* gene. [b] Patient's country of origin

Treatment and replication inhibition (primary culture)

Compound stocks were prepared by dissolution in dimethyl sulfoxide (DMSO) at 10 or 100 milli Molar. Poly-l-lysine (100µg/ml) (Sigma-Aldrich, Bornem Belgium) in Phosphate Buffered Saline (PBS) (Whittaker, Verviers, Belgium) was coated on 96 well flat bottom polystyrene plates (Microtest^{TM}96, Becton Dickinson France S.A., Meylan Cedex, France) at room temperature. After 1 hour plates were washed 3 times with PBS (Bio Whittaker, Verviers, Belgium). To these plates, we added 100 µl of virus at a multiplicity of infection (MOI) of 10^{-4} and 100µl of compound, prepared in 7-fold dilution (final concentration of 100,000 nM - 0.1 nM) in complete medium i.e.

RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 U/ml) and streptomycin (100µg/ml) and 10% Fetal Bovine Serum (FBS) (Biochrom KG, Berlin, Germany). After an incubation period of 1 hour at room temperature, plates were washed thoroughly (6X) with PBS to remove excess compound and unbound virus. The plate-bound and compound-pretreated virus was then seeded with 100µl MO-DC ($4x10^5$ cells/ml) and 100µl autologous resting CD^4+ T cells ($2x10^6$ cells/ml) in complete medium. Each compound concentration was tested in 6 fold. Half of the culture medium (without cytokines and compound) was replaced twice every week during the primary culture phase of 14 days (figure 1).

Rescue of latent virus PHA/IL-2 stimulated PBMCs (Secondary Culture)

Three days before the end of the primary culture, PBMC from a different donor were stimulated with phytohemagglutinin (PHA) at 0.5 µg/ml and IL-2 at 10 U/ml. At day 14 of the primary culture, cells were washed 3X (by centrifugation of the plates at 400*g* for 10 minutes) to remove cell-free virus in the supernatant. The PHA/IL-2 stimulated PBMCs (further referred to as PHA T BLAST) were washed and resuspended at 0.5 x 10^6 cells/ml in complete medium with IL-2 at 10U/ml. Two hundred µl of this suspension was added to all the wells of the DC/T4 cultures for a secondary culture of another 14 days. Half of the culture medium (including IL-2, but without compound) was replaced twice every week.

HIV antigen and EC_{50} of antiretroviral compounds determination

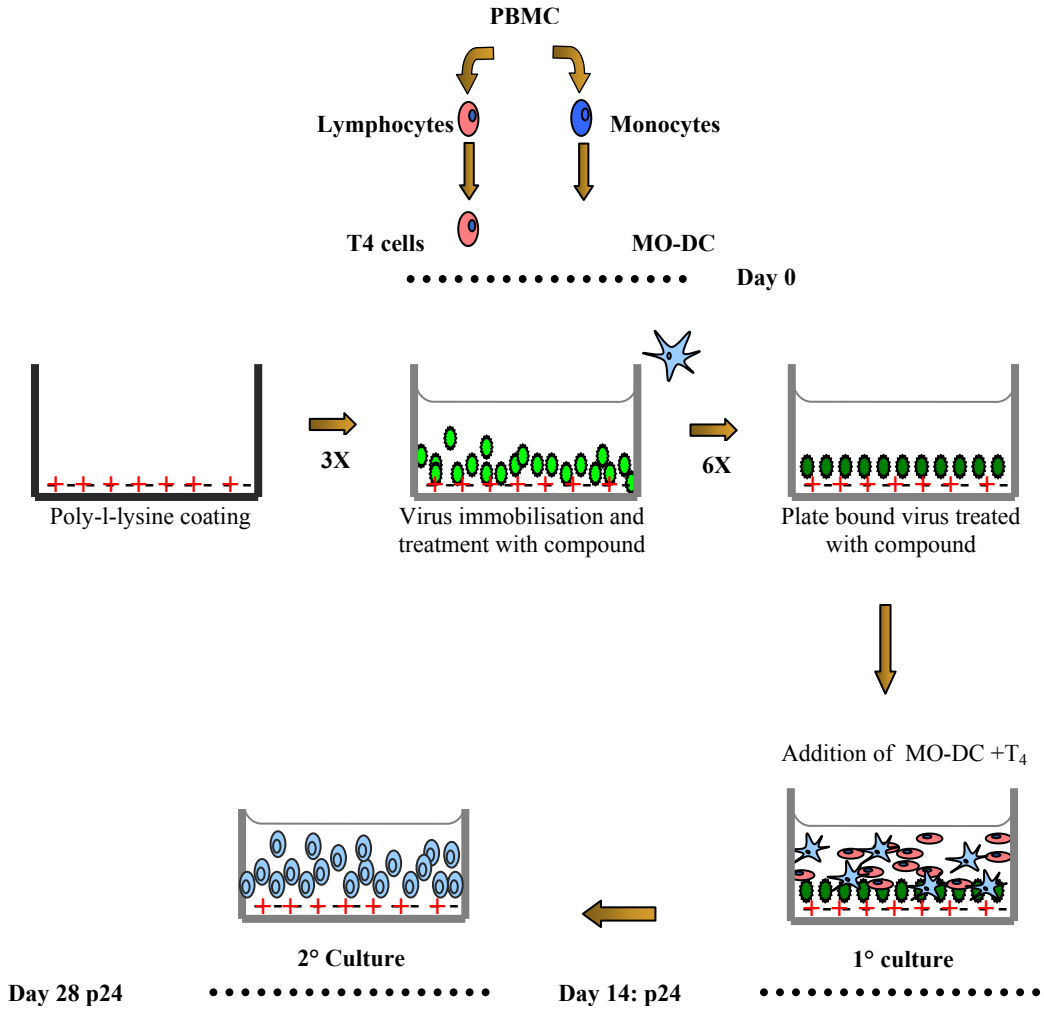
One hundred microlitres of day 7, day 14 (primary culture), day 21 and day 28 (secondary culture) supernatants were collected and inactivated with an equal volume of Nonidet P-40 (NP40) (Calbiochem, EMD Biosciences, Inc. Darmstadt, Germany). HIV antigen in the supernatants was measured using a modified in-house monoclonal p24 (HuMab-HIVp24, Biomaric NV, Ghent, Belgium), enzyme linked immunosorbent assay (ELISA) [23]. Optical density (OD) readings at 450nm were transformed into HIV antigen concentrations, using a standard curve of HIV-1_{Ba-L} stock dilutions, the p24 content of which was previously determined with a commercial kit (Innogenetics).

HIV antigen concentration was plotted against compound concentration (Microplate Manager PC program, BIORAD Laboratories Inc, Hercules, California, USA) and regression analysis was performed on the linear part of the curve to calculate EC_{50} value (equivalent to the compound concentration required to reduce HIV antigen production by 50%).

Quantification of provirus HIV-1 DNA

Provirus DNA was quantified with a commercial kit according to the manufacturer's instructions (Amplicor HIV-1 Amplification kit. Roche Molecular Systems, Branchburg, NJ, USA) [21]. The cut-off value for a negative sample was 10 DNA copies / 1 X 10^6 cells.

Figure 1: Poly-l-lysine based *in vitro* model to study inhibitory capacity of NN-RTIs



Residual compound quantification in supernatant after washing

Several serial dilutions of compound were added to PLL coated plates and left to incubate at room temperature for 1 hr. Plates were then subjected to a series of 6 washing steps and supernatants of the 3rd and 6th wash were collected for analysis. High Performance Liquid Chromatography **(**HPLC) (performed at Janssen Pharmaceutica) was used to quantify the residual compound concentration in the supernatant collected A series of known concentrations of compounds were injected onto the HPLC for detection, the chromatograph of these known concentrations gave a series of peaks that correlated to the concentration of the compound injected and using a computer software (Excel) a liner calibration curve was generated. From this curve unknown concentrations injected in the HPLC were calculated. Supernatant before washing was used as positive control and negative control included complete culture medium.

Residual compound quantification in poly-l-lysine (PLL) coated plates

Radiolabelled ^{14}C-TMC-120 and ^{14}C-AZT were used to evaluate whether compound bound to PLL coated plates and/or associated with cell-free immobilized virus. Plates were coated with 100µg/ml PLL for 1 hour, different concentrations of ^{14}C-compound were added in the presence or absence of cell free HIV virus. Radioactivity in the plates was measured before washing, after 3X washing and after 6X washing, using a scintillation counter (Top count; Canberra-Packard, Zellik, Belgium) and residual amount of compound on plate was expressed as counts per minute (cpm).

Results

Treatment and replication inhibition of HIV-1$_{Ba-L}$ virus

In a series of preliminary experiments we used various infectious doses of the viruses (10^{-3}, 10^{-4} and 10^{-5} MOI) to coat the PLL plates. After washing away the unbound virus and addition of MO-DC and CD4 T cells, it was evident that coating with 10^{-3} and 10^{-4} MOI always resulted in high-level productive infection after 2 weeks, whereas the infection was less consistent at lower concentrations (data not shown). Therefore, we decided to choose 10^{-4} MOI for all further experiments.

Inhibitory capacities of compounds on cell-free HIV-1_{Ba-L} were studied using a wide concentration range (100,000 nM – 0.1 nM) of TMC-120, TMC-125, UC-781, PMPA and AZT. Each compound concentration was duplicated 6 times, incubated with the cell-free virus for 1 hour and thoroughly washed away before addition of DC and CD4 T cells. In the primary DC/T4 culture, pretreatment with TMC-120 and TMC-125 more potently inhibited virus replication than UC-781 by a factor of 11 and 4 respectively (see EC_{50} values in Table 2). The minimal concentration to inhibit HIV-1_{Ba-L} virus production below the detection limit of the ELISA was 100nM for TMC-120, 300 nM for TMC-125 and 1000 nM for UC-781. Remarkably, pre-treatment of the virus with the nucleoside RTI AZT and the nucleotide RTI PMPA, failed to inhibit infection at the highest concentrations used (100,000 nM).

To investigate if any subliminal or latent infection was present in ELISA-negative primary cultures, a secondary culture was initiated by adding PHA T BLAST, known to be the most sensitive target cells for HIV. From cultures, of which the supernatant remained ELISA negative after the secondary culture, cells were additionally checked for the presence of proviral DNA. In fact, if HIV-1_{Ba-L} virus originally had been pretreated with NN-RTIs, very few negative primary cultures turned positive after the secondary cultures.

Treatment and replication inhibition of prevalent HIV-1 group M primary subtypes

Several experiments were performed on the HIV-1_{Ba-L} virus first and then later extended to primary isolates. The inhibitory capacities of the DAPY compounds were similar for the primary isolate panel (see Figure 2). Replication inhibition by the different compounds showed no clear-cut subtype specificity, CI22 (subtype B) was the most susceptible virus to TMC-120, VI882 (subtype C) was the most susceptible to TMC-125 and CI20 (subtype CRF02) was most susceptible to UC-781. In the primary DC/T4 culture, both TMC-120 and TMC-125 were more potent than UC-781 by a factor of 5 (see table 3). The minimal concentration to inhibit all virus clades below the detection limit of the ELISA was between 30-100nM for TMC-120, 10-300 nM for TMC-125 and 1000-3000nM for UC-781.

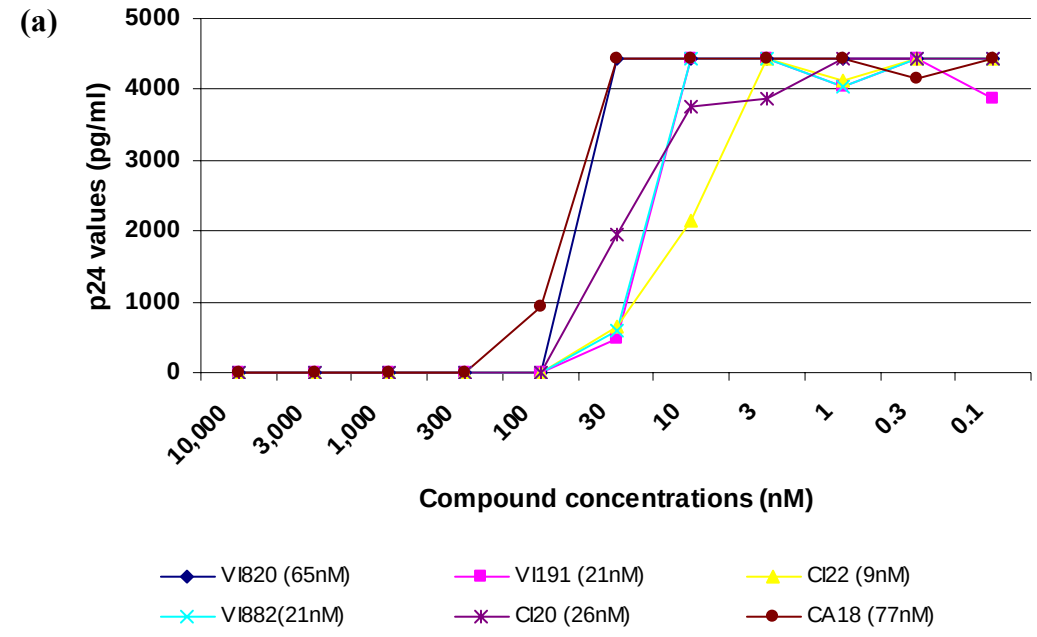
Primary isolates pre-treated with either TMC-120 or TMC-125 showed limited virus rescue in the secondary culture, on the other hand sizeable number of primary cultures from isolates pre-treated with UC-781 turned positive after the secondary cultures. This is reflected in the overall minimal rise of EC_{50} for TMC-120, a slightly higher rise for TMC-125 and more pronounced 5 fold increase for UC-781 (Table 3). An overall comparison of EC_{50} values at the end of the secondary cultures, infected with HIV-1_{Ba-L}, indicates that TMC-120 is 3 times more potent than TMC-125 and 14 times more potent than UC-781. In contrast, on average the six primary isolates were equally sensitive to inhibition by TMC-120 and TMC-125, whereas UC-781 was 30 times less potent.

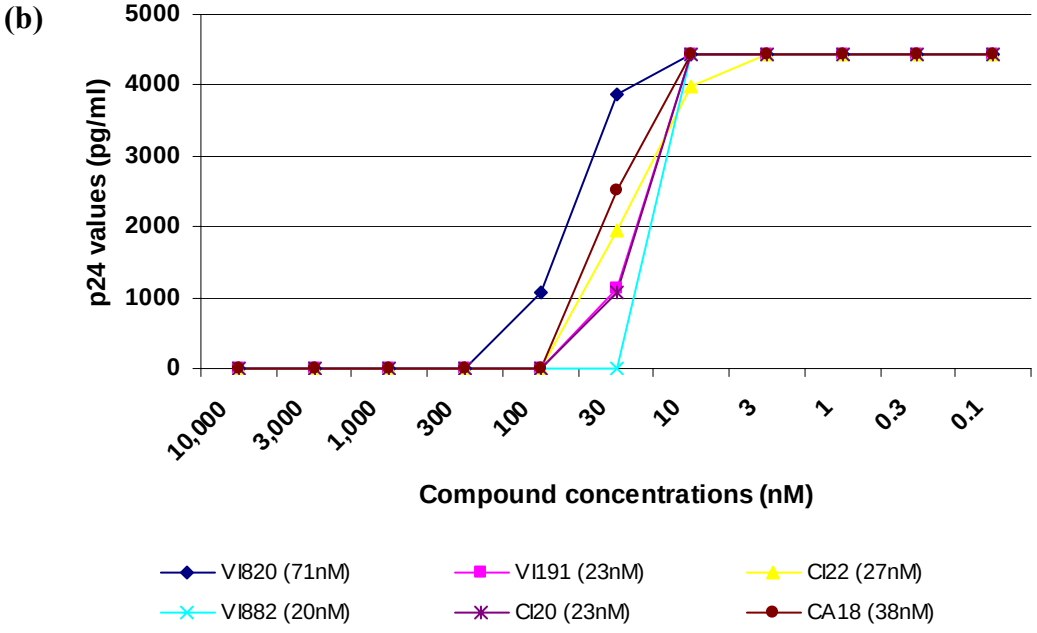
Table 2: Treatment of immobilized cell-free HIV-1_{Ba-L} virus with compounds: Replication Inhibition in primary and secondary cultures.

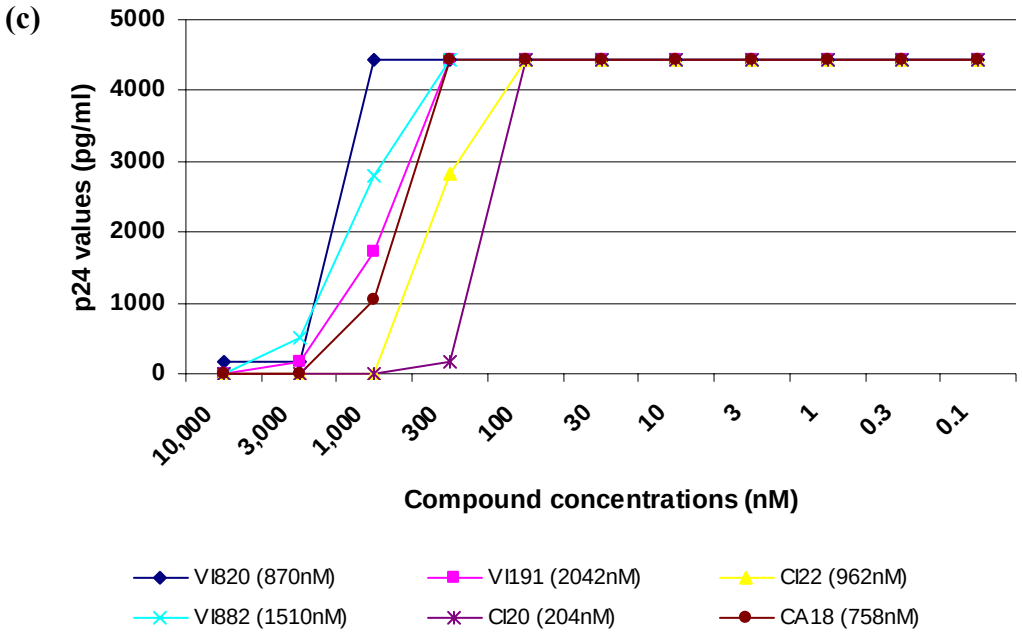
Compound[a]	Class	1° Cul <EC_{50}>(nM)[e]	2° Cul <EC_{50}>(nM)[g]
TMC-120	NN-RTI[b]	37 (± 26[f])	38 (± 26)
TMC-125	NN-RTI	103 (± 86)	117 (± 131)
UC-781	NN-RTI	442 (± 182)	517 (± 193)
PMPA	NtRTI[c]	>100,000	>100,000
AZT	N-RTI[d]	>100,000	>100,000

[a] Cell-fee HIV-1_{Ba-L} (10^{-4} MOI) was immobilized and pre-incubated with compound for 1 hr, washed, seeded with MO-DC and autologous T4 cell and cultured for 2 weeks, without adding compound (primary culture). Day 14 cells are washed (3X) and fresh PHA T BLAST were added and cultured for another 14 days (secondary culture). [b] Non nucleoside reverse transcriptase inhibitor. [c] Nucleotide reverse transcriptase inhibitor. [d] Nucleoside reverse transcriptase inhibitor. [e] EC_{50}: 50% Effective concentration, compound concentration required to inhibit HIV-1 replication in primary culture. [f] Mean ± SD (8 experiments for TMC-120, 6 experiments for UC-781, 4 experiments for TMC-125 and 2 experiments for PMPA & AZT). [g] EC_{50}: 50% Effective concentration, compound concentration required to inhibit HIV-1 replication in secondary culture.

Figure 2: Treatment of immobilized cell-free HIV-1 group M primary isolate viruses, replication Inhibition in **secondary** cultures







Antiviral activity of TMC120(**a**), TMC125(**b**) and UC781(**c**) on six primary isolates in secondary co-cultures. A serial dilution of compound was added to immobilized virus (10^{-4} MOI), excess compound was washed away and target cells added. Culture was kept for 14 days, a secondary culture was initiated for a further 14 days. HIV antigen (p24) was measured in the supernatants by ELISA and is represented as picograms per milliliter. Fifty percent effective concentration compound concentration (EC_{50}), calculated in nanomolar (nM) by linear regression analysis. Subtype information of the viruses used are as follows: VI820 (subtype A), VI191 (subtype A/G), CI22 (subtype B), VI882 (subtype C), CI20 (subtype CRF02_AG), and CA18 (subtype CRF02_AG).

Residual compound in supernatant, bound on plates or virus

In order to know if any compound (TMC-120 and TMC-125) could remain present after pretreatment of the cell-free virus and extensive washes, the supernatants after 6^{th} wash were subjected to high-pressure liquid chromatography (lower detection limit of 8nM). No traces were detectable even if the highest concentration 10,000nM was used (data not shown).

In addition, we evaluated residual plate-bound TMC-120 and AZT, using ^{14}C-labeled compounds. At inhibitory concentrations of TMC-120 (10-30nM), less than 0.22 % of ^{14}C-TMC-120 is bound to plate (in the absence of virus). At concentrations well above the inhibitory capacity of the TMC-120 (100nM -10,000nM) an average of 2.7 % of ^{14}C-TMC-120 is bound to the plate (data not shown). There was no difference in residual ^{14}C-TMC-120, in the presence or absence of virus. In contrast ^{14}C-AZT could be washed away completely from the PLL coated plate with or without virus.

Table 3: Treatment of immobilized cell-free HIV-1 group M primary isolates virus with compounds: Replication Inhibition in primary and secondary cultures

	TMC-120[a]		**TMC-125**		**UC-781**	
	1° CuI (nM)	**2° CuI(nM)**	**1° CuI (nM)**	**2° CuI (nM)**	**1° CuI (nM)**	**2° CuI (nM)**
VI820	63.5[b]	65.0[c]	13.4	71.1	261.4	870.9
VI191	20.9	21.2	<10.0	23.5	588.3	2042.0
CI22	7.1	9.8	18.9	27.3	192.2	962.1
VI882	19.0	21.6	11.8	20.0	67.5	1510.9
CI20	20.7	26.9	18.9	23.2	34.4	204.3
CA18	74.4	77.2	<10.0	38.1	84.0	758.2
<EC_{50}> ±SD	34 (±25)	36 (±24)	14 (±4)	33 (±17)	204 (±188)	1058 (±582)

[a] Cell-fee primary isolates (10^{-4} MOI) was immobilized and pre-incubated with compound for 1 hr, washed, seeded with MO-DC and autologous T4 cell and cultured for 2 weeks, without adding compound (primary culture). Day 14 cells are washed (3X) and fresh PHA T BLAST were added and cultured for another 14 days (secondary culture). [b] EC_{50}: 50% Effective concentration, compound concentration required to inhibit HIV-1 replication in primary culture. [c] EC_{50}: 50% Effective concentration, compound concentration required to inhibit HIV-1 replication in secondary culture.

Discussion

We evaluated the direct effect of various reverse transcriptase inhibitors on immobilized cell-free virus and clearly show that all NN-RTIs have the capacity to completely prevent subsequent cellular infection (at nanomolar and micromolar concentrations respectively).

This pre-treatment model mimics the scenario where a drug acts on the virus before it can enter the epithelial layer and reach the subepithelial target cells (represented in our model by MO-DC and CD4 T cells). The six primary isolates (HIV-1 group M subtypes A, A/G, B, C and CRF02_AG) used in this study were mostly from seropositive African individuals and represent the relevant circulating subtypes. Primary HIV-1 group M isolates were similarly sensitive as HIV-$1_{B\text{-}aL}$ to inhibition by NN-RTI. The fact that sterilizing capacities of the NN-RTI tested showed no subtype specificity strongly suggests, but obviously does not prove that they could be used as a sterilizing microbicide.

The diarylpyrimidine compounds TMC-120 and TMC-125 were consistently more potent than the structurally unrelated carboxyaniline UC-781. In addition, viral rescue was infrequently observed when primary isolates were pre-treated with the DAPY compounds, but occurred more after UC-781 pre-treatment. Collectively, these data indicate that DAPY compounds inactivate RT enzymatic activity in a more efficient manner than UC-781, hence preventing the virus from being integrated in the host cell genome.

A remarkable observation was that PMPA, a classic nucleotide RTI, and AZT, a nucleoside RTI, failed to inhibit virus. In previous studies PMPA and AZT have been shown to efficiently abort HIV infection when they remain present during the first 24 hours of the cultures of MO-DC and CD4 T cells, with EC_{50} value of 440nM and 25nM respectively [16]. This apparent discrepancy can be explained by the fact that Nt-RTI and N-RTI need cellular activation to display RTI activity whereas the DAPY compounds (NN-RTIs) are directly inhibitory on viral RT [3].

We observed that a small percentage of the highly hydrophobic DAPY compounds was sticking to the wells, despite extensive washing. Therefore, it is not excluded that the residual compound, bound to the plate prolongs the pre-treatment effect. This "stickiness" could also be an advantage in vivo, as demonstrated by Shattock's experiments. When a cervical explant tissue was treated with TMC-120 or UC781 for 2 hour, HIV infection was blocked up to 6 days after exposure to compound, creating a significant anti-HIV "memory effects" [25].

In conclusion our in vitro model allows rapid screening of direct anti-viral effects on cell-free HIV in a system using primary HIV-1 isolates and primary MO-DC/T4 cells, closely mimicking targets, involved in HIV transmission. The new DAPY compounds, TMC-120 and TMC-125 show high antiviral activity against different viral clades (predominant in the epidemic), suggesting that both compounds could be good candidate microbicide. However, in the absence of any microbicide of proven potency in women, no direct conclusion can be drawn from any *in vitro* system and careful preclinical testing in animal models of candidate microbicides remains mandatory.

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Non nucleoside reverse transcriptase inhibitors as virucidal microbicides – Is there a modulation in function of the target cells?

Antiviral Chemistry and Chemotherapy (*submitted*)

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Summary

Second generation non nucleoside reverse transcriptase inhibitors (NNRTIs) are possible candidates for microbicides but due to the small size and the high lipophylicity of the molecule, it is likely that NNRTIs when used as tropical microbicides will be absorbed through the vaginal or endocervical mucosa. In this study we investigated the immune suppressive capacities of these compounds on T cell responses to flu antigen, presented by monocyte derived dendritic cells (MO-DC).

Co-cultures of (MO-DC) and autologous CD4(+) T cells in the presence of a recall Flu antigen and compound were incubated for 6 days, afterwards, ^{3}H-Thymidine was added to each well. Cells were harvested after 7 hours and incorporated radioactivity measured in a scintillation counter. In addition, using a recently described *in vitro* system, based on co-cultures of dendritic cells and resting CD4(+) T cells the antiviral activity of the NNRTIs on cell-free HIV_{Ba-L} was determined.

With prolonged exposure (7 days), TMC120 ($ISC_{50=}$1,065nM) was the most immune suppressive followed by UC781 ($ISC_{50=}$13,946nM) and TMC125 ($ISC_{50=}$24,609nM). Under continuous treatment of compound, TMC-120 and TMC125 showed the most potent antiviral activity and inhibited replication of HIV-1_{Ba-L} with an average EC_{50} value of 1.35nM (SD ± 1.62) and 74.5nM (SD ± 28.0) respectively. UC781 was the least potent with an EC_{50} of 87.0 nM (SD ± 28.0). In conclusion, the tested DAPY compounds have highly different immune suppressive profiles but show a similar wide range of therapeutic index in vitro.

Introduction

The concept to use reverse transcriptase inhibitors (RTI) as topical microbicides was first considered following the demonstration of the virucidal effects of UC781, a thiocarboxanilide non-nucleoside reverse transcriptase inhibitor (NNRTI) (Borkow et al., 1997). New classes of even more potent NNRTIs are being developed e.g. the diarylpyrimidines (DAPY) analogues. Two of these, TMC120 (dapivirine) and TMC125 (etravirine), show anti-HIV activity on 80% of clinical strains with typical mutations for NNRTI resistance, with an EC_{50} below 0.1µmol/l (De Bethune et al., 2001; Andries et al., 2004).

In a recent study (Njai et al., 2005), the microbicidal activity of TMC120, TMC125 and UC781 were evaluated in an in vitro model of MO-DC + CD4(+) T cells. Pre-treatment of cell-free HIV primary isolates for at least 1 hour with compound potently inhibited viral replication at micro molar concentrations. A gel formulation of TMC120 was used in an *in vivo* model of SCID mice and prevented infection of cell-associated HIV by 70-80% at concentrations as low as 2.25 micro molar (Di Fabio et al., 2003). Due to the small size and the high lipophylicity of the molecules, it is likely that NNRTIs when used as tropical microbicides will be absorbed through the vaginal or endocervical mucosa. Hence we propose to investigate the possible interference of these compounds with antigen processing by mucosal dendritic cells.

Method

Generation of monocyte derived dendritic cells (MO-DC) and CD4(+) T cells.

Monocytes and lymphocytes were purified from HIV negative buffy coats. The former were cultured in complete medium i.e. RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 U/ml) and 2.5% Human Serum (HuS) with granulocyte-monocyte colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4) to induce DC differentiation. CD4(+) T cells were purified from the cryopreserved lymphocytes. The protocol was based on Sallusto et al (1994), with modifications previously described (Vanham et al., 2000).

Immune suppressive activity of compound on antigen driven-cell proliferation.

Autologous co-cultures of monocyte derived dendritic cells (MO-DC) and CD4(+) T cells were incubated with 10µg/ml of recall flu antigen (Influenza Virus Antigen A/Sydney/5/97 (H3N2) (Resvir-13) NIBSC, Potter Bars, Herts, U.K) in the presence of compound in complete medium i.e. RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 U/ml) and 2.5% Human Serum (HuS). Seven compound concentrations ranging from 100,000nM – 100nM were tested in six replicates. MO-DC + CD4(+) T cells with antigen but without compound was used as positive control. The cultures were incubated for 6 days in an incubator (37 °C and 5% CO_2). On day six, 1 µCi of [methyl- ^{3}H]-Thymidine (Tra.120 Amerischam Pharmacia, Buckingham, UK) was added to each well and further incubated for 7 hours. Cells were then harvested on glass filter paper and incorporated radioactivity was measured in a scintillation counter (Top count; Canberra-Packard, Zellik, Belgium) and expressed as counts per minute (cpm). The immune-suppressive concentration (ISC_{50}) is defined as the compound concentration inhibiting 50% of CD4(+) T lymphocyte proliferation.

Inhibition of cell-free HIV-1_{Ba-L} replication under continuous treatment of compound.

NSI/R5, monotropic strain HIV-1_{Ba-L} was kindly provided by the National Institute of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD, USA). The 50% tissue culture infectious dose ($TCID_{50}$) was determined by titrating virus stock in MO-DC and CD4(+) T cell co-cultures. Cell free HIV-1_{Ba-L} was pretreated with NNRTI as previously described (Njai et al., 2005). Briefly, poly-l-lysine (100µg/ml) (Sigma-Aldrich, Bornem Belgium) in Phosphate Buffered Saline (PBS) (Whittaker, Verviers, Belgium) was coated on 96 well flat bottom polystyrene plates (Microtest^{TM}96, Becton Dickinson France S.A., Meylan Cedex, France) at room temperature. After 1 hour plates were washed 3 times with PBS (Bio Whittaker, Verviers, Belgium). To these plates, we added 100 µl of virus at a multiplicity of infection (MOI) of 10^{-4} and 100µl of compound, prepared in 7-fold dilution in complete medium i.e. RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 U/ml) and 2.5% Human Serum (HuS).

Each compound concentration was tested in 6 replicates. After an incubation period of 1 hour at room temperature, plates were washed thoroughly (6X) with PBS to remove excess compound and unbound virus. The plate-bound and compound-pretreated virus was then seeded with 100µl MO-DC ($4x10^5$ cells/ml) and 100µl autologous resting CD4+ T cells ($2x10^6$ cells/ml) in complete medium and cultured for 7 days. Half the culture medium was replaced twice with compound in complete medium.

HIV antigen and EC_{50} of antiretroviral compounds determination.

One hundred microlitres of day 7 supernatant was collected and inactivated with an equal volume of Nonidet P-40 (NP40) (Calbiochem, EMD Biosciences, Inc. Darmstadt, Germany). HIV antigen in the supernatants was measured using a modified in-house monoclonal p24 (HuMab-HIVp24, Biomaric NV, Ghent, Belgium), enzyme linked immunosorbent assay (ELISA) (Beirnaert et al., 1998). Optical density (OD) readings at 450nm were transformed into HIV antigen concentrations, using a standard curve of HIV-1_{Ba-L} stock dilutions, the p24 content of which was previously determined with a commercial kit (Innogenetics). HIV antigen concentration was plotted against compound concentration (Microplate Manager PC program, BIORAD Laboratories Inc, Hercules, California, USA) and regression analysis was performed on the linear part of the curve to calculate EC_{50} value (equivalent to the compound concentration required to reduce HIV antigen production by 50%).

Results and Discussion

An ideal microbicide will block infection in an early stage, at least before integration of the virus in the host cell; it must have high activity against different viral clades, combined with a low toxicity and irritation potential. Several recent studies (Njai et al., 2005; Van Herrewege et al., 2004; Andries et al., 2004) have shown the potent antiviral activities of second generation non-nucleoside reverse transcriptase inhibitors NNRTIs (TMC120, TMC125 and UC781) on different strains of viruses and implied their use as possible topical microbicides. However little is known about their modulation effect (if any) in function of the target cells.

Epithelial Langerhans cell or interstitial-type DCs are the first targets for HIV during sexual transmission (Masurier et al., 1998; Romani et al., 1994). Both cell types express the classical HIV receptor CD4 and the chemokine-co-receptor CCR5. The recently discovered DC-SIGN, however, is selectively expressed on the interstitial DC. This integrin-like molecule seems critically involved in HIV binding, protection from degradation and transfer to T4 cells. Our in vitro model on monocyte-derived DC (MO-DC) used in this study resemble more closely interstitial DC rather than Langerhans cells. In previous papers, we and other showed that incubation of MO-DC with cell-free (or cell-associated) NSI/R5 HIV and co-culture with resting autologous CD4 T cells results in a productive infection, without the need for added cytokines (Njai et al., 2005; Van Herrewege et al., 2004; Vanham et al., 2004).

In vitro functional responses can be variable even within a given donor, hence for each experiment we used a different donor in order to derive robust estimate of the concentration of compound needed for 50% inhibition of T cell proliferation. In the immune suppressive experiments, autologous co-cultures of monocyte derived dendritic cells (MO-DC) and CD4(+) T cells were incubated with antigen and compound for a period of 7 days. TMC120 was the most immune suppressive compound with an ISC_{50} values of 1,065 nM, and the least immune suppressive was TMC125 (ISC_{50} = 24,609nM) (See Figure 1). Remarkably TMC125, which differs structurally from TMC120 by a few atoms on its side chains, shows a better immune suppressive profile, at least 23 fold less immune suppressive than TMC120. UC781, a structurally different NNRTI had an ISC_{50} value of 13,946nM. Since the compound was present all through the culture, the effects observed might be a cumulative effect on antigen-processing and presentation, as well as on the T cell response.

Even though the antiviral activities of these compounds have been studied before (Njai et al., 2005; Van Herrewege et al., 2004; Andries et al., 2004), to predict a better therapeutic we evaluated the blocking effect on the laboratory strain HIV-$1_{Ba\text{-}L}$ under continuous treatment of compound. TMC-120 inhibited replication of HIV-$1_{Ba\text{-}L}$ with an average EC_{50} value of 1.35nM (SD ± 1.62).

TMC125 was the second most potent compound and inhibited replication of HIV-1_{Ba-L} with an EC_{50} value of 74.5nM (SD ± 28.0). UC781 the least potent compound showed and EC_{50} of 87 nM (SD ± 31.1).

The therapeutic index of a compound is defined as the ratio of the toxic dose to the therapeutic dose. We calculated the therapeutic indexes of all the compounds tested and showed that TMC120 had the largest therapeutic index even though it was the most immune suppressive. TMC120 (TI = 760) had a therapeutic index 2.3 fold larger than TMC125 (TI = 330) and 4.8 fold higher than UC781 (TI = 160).

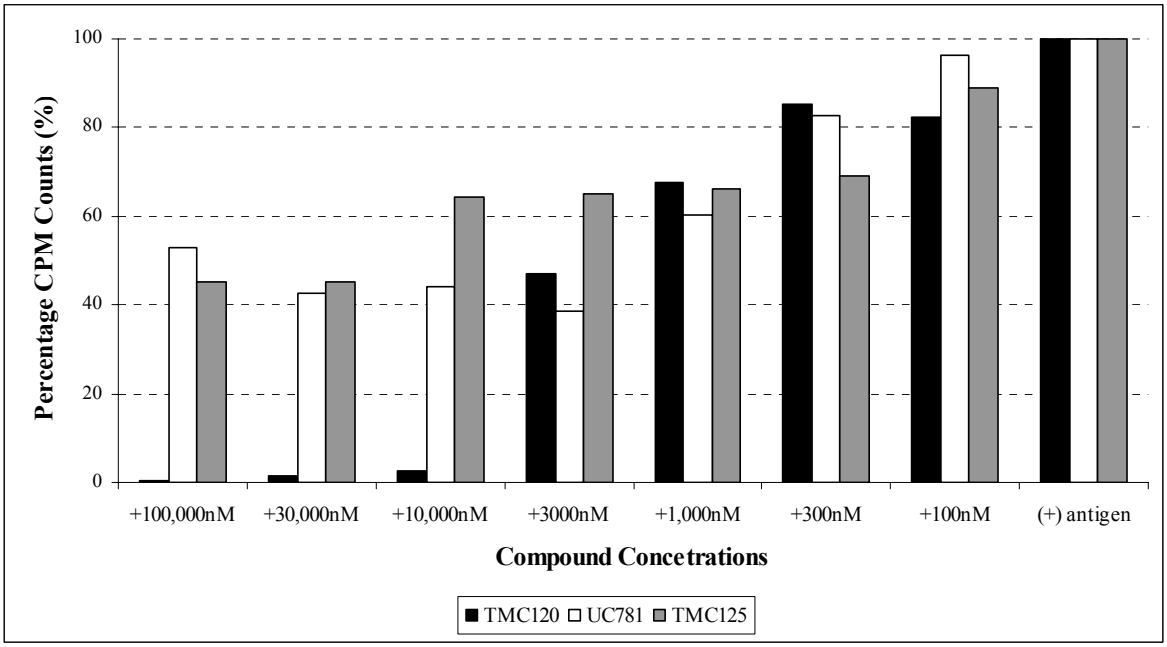
In conclusion, the tested DAPY compounds have highly different immune suppressive profiles but show a similar wide range of therapeutic index in vitro. However, TMC120 concentrations, shown to be effective, when applied vaginally in SCID mice (Di Fabio et al., 2003), are well within the immune micromolar suppressive range. Therefore, one needs to carefully measure the absorbed fraction in order to assess whether local or systemic immune suppression could present a problem.

Table 1: Continuous treatment of immobilized cell-free HIV-1_{Ba-L} virus with compounds: Replication Inhibition in 7 day cultures

Compound [a]	**EC_{50} value (nM)** [b]	**ISC_{50} value (nM)** [d]	**Therapeutic Index** [e]
TMC-120	1.4 (±1.6) [c]	1,065	760
TMC-125	74.5(±28.0)	24,609	330
UC781	87.0 (±31.1)	13,946	160

[a] Cell-fee HIV-1_{Ba-L} (10^{-4} MOI) was immobilized and pre-incubated with compound for 1 hr, washed, seeded with MO-DC and autologous T4(+) cell and cultured for 1 week, half the culture medium was replaced twice with complete medium and compound. [b] EC_{50}: 50% Effective concentration required to inhibit HIV-1 replication. [c] Mean ± SD (average of 3 experiments for each compound). [d] ISC_{50}: 50% Effective concentration required to inhibit 50% of CD4 T lymphocyte proliferation. [e] TI, Therapeutic Index (ISC_{50}/EC_{50})

Figure 1: Immune suppressive activity of NNRTIs (TMC120, TMC125 and UC781) on antigen driven cell proliferation.



Immune suppressive activity of TMC120, TMC125 and UC781 on antigen driven cell proliferation. The set consists of an autologous co-culture of monocyte derived dendritic cells (MO-DC) and T_4 cells in the presence of a recall Flu antigen with or without compound (different concentrations). Methyl- ^{3}H-Thymidine incorporation after 7 days was expressed as counts per minute (cpm). Results indicated represent averages of 2 experiments for each compound. A different buffy coat was used in each experiment to generate target cells.

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4.1. General discussion

Many of the circulating HIV strains around the world appear to have arisen through recombination and it is likely that recombination may be an important mechanism by which new HIV variants are created that are better adapted to their host e.g. to evade drug or immune pressures [1]. Recombination requires the simultaneous infection of a cell with two different proviruses, allowing the encapsidation of one RNA transcript from each provirus into a heterozygous virion. After the subsequent infection of a new cell, the reverse transcriptase, by jumping back and forth between the two RNA templates, will generate a newly synthesized retroviral DNA sequence that is recombinant between the two parental genomes [2, 3].

Recombinant strains of HIV-1 have been observed worldwide [4-8]. To date there are 18 Circulating Recombinant Forms (CRFs) of which three, CRF01_AE, CRF02_AG and CRF03_AB are of epidemiological importance. CRF01_AE [5, 9, 10], and CRF02_AG [11-13] are causing heterosexual epidemics in Asia and West and West Central Africa, respectively. Circulation of CRF03_AB [14, 15] in Russia, appears to have been accelerated by intravenous injection of a locally produced opiate contaminated with HIV infected blood. CRF02_AG plays an important role in the epidemic in West Africa, where it caused 31% of all new HIV-1 infections. This recombinant form was also reported in West Central and East African countries. CRF02_AG is associated with rapidly spreading new epidemics in western African countries where until recently this epidemic was maintained at relatively low level.

To date, there have been few systematic large-scale attempts to characterize HIV isolates, and especially CRFs, emerging from different parts of the world. As such, our knowledge of the distribution of HIV strains in different populations and about changes in that distribution over time is rather limited. Sequencing is the gold standard of subtyping but due its high cost and difficulty to transfer to poor resource settings, there are low numbers of full-length genome sequences from many areas.

Other subtyping methods are available e.g. gag and env Heteroduplex Mobility Assay (HMA), but the close genetic distance between subtype A fragments in CRF02_AG and subtype A strains hampered differentiation between CRF02_AG and subtype A by *env* HMAs and alternate experimental conditions are needed to discriminate between subtype A, CRF01_AE, and CRF02_AG by *gag* HMA [16].

A major challenge in the design and evaluation of efficacious subtype-dependent candidate HIV-1 vaccines is the development of techniques for large-scale HIV genetic characterization to document the true prevalence rates of HIV subtypes and CRFs in developing countries. Here we describe the design and potential use of a CRF02_AG-specific oligonucleotide probe hybridization assay for large-scale monitoring of the prevalence of CRF02_AG variants (**Chapter 3 – Section 3.1**). The newly developed oligonucleotide probe hybridization assay showed high signal reactions to probes, differentiating CRF02_AG from other subtypes (A to H) and CRFs (CRF01 and CRF06). The oligonucleotide probe hybridization assay was validated and had a sensitivity of 98.4%, a specificity of 96.7%, a positive predictive value of 98.4%, and a negative predictive value of 96.7%, which make the assay very reliable.

The probe assay as a diagnostic tool will allow rapid screening for CRF02_AG; this could be used for tracking of the HIV epidemic in terms of documenting the real prevalence of CRF02_AG virus infections. The assay is easy to use and can be easily transferred to poor resource setting. Hence the assay could be a rapid and economical tool for use in the large-scale screening of this particular subtype at vaccine trial sites, especially in West Africa. The high sensitivity and specificity of the test also imply that there will be high positive and negative predictive values in regions of high prevalence (pre-existing CRF02_AG epidemic) and low prevalence (new epidemics of CRF02_AG).

Subtype-specific probes can be designed on the basis that an insertion / deletion pattern compared to other subtypes and CRFs is conserved. The five CRF02_AG probes were designed based on the conserved deletion pattern found in the p17 gag region of the CRF02s only.

Designing subtype-specific probes based on difference in point mutations can be challenging. This could mean regular extension, evaluation and updating of the probe panel used leading to a lot more probes to be designed, and then the probe assay will be expected to be expensive. A subtype C probe assay was designed based on differences in point mutation with disappointing sensitivity and specificity [17].

The emergence of CRF02_AG as the predominant strain causing HIV infections in West Africa may simply be a chance event. However, the fact that CRF02_AG has spread much more rapidly than other clades (including clade A and G) and other CRFs in West and West Central Africa, where many HIV-1 group M subtypes as well as group O and HIV-2 viruses co-circulate, raises concern that CRF02_AG may be favoured, in terms of superior replication fitness and/or transmission efficiency. In **Chapter 3 – Section 3.2** we tested the hypothesis that the predominance of CRF02_AG recombinant in West Central Africa is related to a higher replicative fitness. Our data clearly indicated that CRF02_AG isolates had a significant replication advantage over its parental subtypes. Higher fitness displayed by the CRFs could lead to increasing prevalence of the recombinants in regions where CRF already circulates.

It is always presumed that recombination is a mechanism that an organism uses to rapidly improve its adaptation to the prevailing environment. Thus many “unique recombinant form” (URF) have been found in individuals, who apparently were infected with viruses from two different subtypes. Apparently, this particular inter-subtype recombinant form (IRF) was better adapted to this particular patient than the original subtypes. Recombination also plays a role in adaptation to drug pressure: multi-resistant viruses are generated by recombining pre-existing viruses with one or few mutations each. Of all possible URF only 18 have been shown to spread in an epidemic fashion. Until now, one could think that all CRF epidemics were the consequence of founder effects.

The data from this thesis is the first experimental evidence that the spread of at least this CRF, which occurs in an area with many other co-circulating subtypes, is not just coincidence but that CRF02 indeed has a competitive advantage over the others.

This interpretation is consistent with the observation by Ariën et al [18] from our laboratory, who previously showed that group M viruses in general have a much higher in vitro fitness than group O or HIV-2 viruses, corresponding to the relative spread of these virus groups in the pandemic as a whole and in West Africa (where they all co-circulate) in particular.

Clearly, our most convincing data on the competitive advantage of CRF02 were generated in activated T cells, which we consider a model of "pathogenic fitness". The finding is consistent with the observation of Fischetti, showing higher viral load in CRF02 infected individuals [19]. Clinical-epidemiological studies in the same population, however, do not show a difference in disease progression between CRF02 and non-CRF02 infected subjects [20]. Fitness differences in dendritic cells, which we consider as a model of transmission, were less significant.

Finally, detailed analysis of West African viruses shows that CRF02 has already undergone further recombination. This process may be enhanced if the virus spreads over the rest of the world [21-24] and encounters other subtypes that are also successful, e.g. subtype C, co-infecting host with different genetic backgrounds [25]. Viral evolution will continue and a CRF that we label as very fit today, may be out competed by a more complex and even fitter recombinant tomorrow.

Women are at a greater risk of acquiring Human Immunodeficiency Virus (HIV) than men [26] and they account for nearly 50% of all people living with HIV worldwide [27]. In some parts of Africa, the incidence of HIV is alarmingly high in younger women (age 15-25). Even though effective preventive measures (condoms) exist they are often not a feasible option for women due the need of consent from male partners. In view of this situation, a new focus in prevention is on the development of microbicide that, when applied topically, should substantially reduce transmission of HIV. An ideal microbicide should be able to block infection in an early stage, at least before integration of the virus in the host cell DNA. Clinical trails are likely to be carried out in Africa where prevalence of HIV is high, hence it is important to know if candidate microbicide can equally inhibit relevant circulating HIV strains, including CRF02_AG.

This inspired us to study the inhibitory effect of various reverse transcriptase inhibitors (RTI) on cell-free HIV in co-cultures of dendritic cells and resting CD4 T cells, modelling early target cells during sexual transmission (**Chapter 3 – Section 3.3)**. The compounds tested included the second-generation non-nucleoside reverse transcriptase inhibitors (NNRTI) TMC-120 (R147681, dapivirine) and TMC-125 (R165335, travertine) as well as the reference nucleoside RTI AZT (zidovudine), the nucleotide analogue PMPA (tenofovir) and the non-nucleoside RTI UC-781. This pre-treatment model mimics the scenario where a drug acts on the virus before it can enter the epithelial layer and reach the subepithelial target cells (represented in our model by MO-DC and CD4 T cells).

The six primary isolates (HIV-1 group M subtypes A, A/G, B, C and CRF02_AG) used in this study were mostly from seropositive African individuals and represent the relevant circulating subtypes. Primary HIV-1 group M isolates were similarly sensitive as HIV-$1_{B\text{-}aL}$ to inhibition by NN-RTI. TMC-120 and TMC-125, belonging to the novel classes of NNRTI called the Diarylpyrimidine (DAPYs) were clearly more potent than the reference NNRTI. These data suggest that NN-RTIs inactivate RT enzymatic activity of different viral clades (predominant in the epidemic) and might be proposed for further testing as a sterilizing microbicide worldwide.

In conclusion, this study showed an excellent overall susceptibility of different viral clades, from drug-naive seropositive individuals to second generation NNRTIs. These second generation products remain active against viruses that are resistant to first generation NNRTI, including Nevirapine. This is an important feature, since Nevirapine has been used as a monotherapy in mother-to-child transmission (with proven high levels of resistance induction) and in first line HAART, where it also easily induces resistance.

Due to the small size and high lipophilicity of NNRTIs, it is possible that these molecules when used as topical microbicide would be absorbed through the mucosa. The extent of absorption should be carefully controlled in order to avoid two possible unwanted consequences: development of resistance or immune suppression.

With regard to the former, ideally microbicides should be freely available to all women, who consider themselves at risk for acquiring HIV. No pre-testing should be required, because it will increase the threshold for use. Clearly, inevitably, seropositive women, who are unaware of their status, will also use the product and thus resistance might be induced. It is presently unclear how big the chances are on this scenario in real life. It will depend on the frequency of use, the degree of persistence and penetration etc. Therefore, ideally, monitoring should be done to evaluate if the problem occurs and, by preference, products used as microbicides and those for systemic therapy should not show a pattern of cross-resistance

In **chapter 3 – Section 3.4** we investigated the immune suppressive capacities of second generation NNRTIs on T cell responses to flu antigen, presented by monocyte derived dendritic cells (MO-DC). In parallel the antiviral activities of the NNRTIs were determined under continuous treatment of compound and the therapeutic index calculated. With prolonged exposure (7 days), TMC120 was the most immune suppressive followed by UC781 and TMC125.

However the DAPY compounds had antiviral activity against HIV replication well below the immune suppressive concentrations. The DAPY compounds tested have highly different immune suppressive profiles but show a similar wide range of therapeutic index in vitro. A successful microbicide could be marketed as a gel to be applied topically or inserted in a vaginal ring where there will be continual release of the product. TMC120 concentrations, shown to be effective, when applied vaginally in SCID mice [28], are well within the immune micro molar suppressive range. Therefore, one needs to carefully measure the absorbed fraction in order to assess whether local or systemic immune suppression could present a problem.

4.2. Future Perspectives

Recombination has proven to be an important strategy of retroviral adaptation and our observations suggest that recombination could generate HIV variants with increased replication capacity and transmission efficiency. Yet, conceptually, one expects that virus variants with a relatively high transmission capacity and a relatively low pathogenic potential will be most favoured for transmission and hence become most prevalent in a particular human population. To elucidate this question, more work needs to be done in these areas:

Track the spread of CRF02_AG

Based on improved techniques to distinguish CRF02 from other variants (such as the oligonucleotide probe hybridization assay, we developed) the relative speed of the CRF02 epidemic should be monitored in Africa and elsewhere, to ascertain whether this recombinant indeed has a transmission advantage in vivo.

Develop a better in vitro transmission model

Our transmission model (MO-DC) data was not significant, perhaps, a better in vitro models should be developed to mimic the physiology of sexual transmission e.g. by including Female Genital Track (FGT) epithelia and other important target cells, such as T cells and macrophages, in addition to DC. In this way, a better in vitro correlate of transmission fitness could be established.

Investigate the determining factor for the increased transmission fitness of the CRF02_AG

The CRF02_AG genome is a mosaic of subtype A (gag, vpr and parts of pol, env and nef) and subtype G (LTR, rev, tat and parts of pol, env, and nef). It would be interesting to determine which part of the CRF genome is the determining factor for the increased transmission fitness. One way to investigate this question is to create artificial recombinants, by selective exchange of “pure” subtype genes with their CRF homomlogue and competing each of these artificial “chimeric” recombinants against the paternal virus.

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