

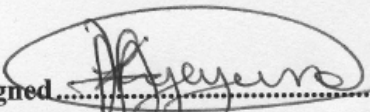
**GENOTYPIC ANTIRETROVIRAL DRUG RESISTANCE IN
A COHORT OF HIV INFECTED DRUG NAÏVE UGANDAN
WOMEN**

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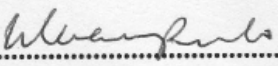
**A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF
MASTER OF SCIENCE, PHARMACOLOGY DEGREE, MAKERERE UNIVERSITY.
MARCH 2010**

DECLARATION

I hereby declare that the work presented in this thesis is my own and has not been submitted for the award of a degree in any other University

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DEDICATION

To all those that have endeavored to ensure that I get education

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Abbreviations and acronyms

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired Immunodeficiency Syndrome
APOBEC3F	Apolipoprotein B (apoB) mRNA-editing enzyme catalytic polypeptide-like 3GF
APOBEC3G	Apolipoprotein B (apoB) mRNA-editing enzyme catalytic polypeptide-like 3G
ART	Anti-retroviral therapy
ARV	Anti-retroviral
AZT	Zidovudine
CA	Capsid Protein
CCR5	Chemokine receptor 5
CD4	Cluster of differentiation 4
CRF	Circulating Recombinant Forms
CXCR4	Chemokine (CXC motif) receptor 4
D4T	Stavudine
DC-SIGN	Dendritic cell specific C type lectin
DDC	Zalcitabine
DDI	Didanosine
DMPA	Depot Medroxyprogesterone Acetate
DNA	Deoxyribonucleic acid
DNTP	Dinucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid
ENV	Envelop gene
ENV	Envelop
Gag	Group Specific Antigen
GP 41	Glycoprotein 41
GP	Glycoprotein
GP120	Glycoprotein 120
GS	Genital Shedding study
HAART	Highly Active Antiretroviral Therapy
HC-HIV	Hormonal Contraception and the Risk of HIV Acquisition
HIV	Human immunodeficiency virus
HMG(Y)	High mobility group DNA-binding cellular protein
HR	Heptad repeats
IAS	International AIDS Society
IRB	Institution Review Board
LTR	Long Terminal Repeats
MP	Matrix Protein
MAP	Mitogen-activated protein
MgCl ₂	Magnesium Chloride
MHC	Major Histo Compatibility Complex
mM	millimolar
NC	Nucleocapsid

NEF	Negative Factor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PI	Protease Inhibitor
PIC	Preintegration Complex
POL	Polymerase gene
RANTES	Regulated upon activation normal T expressed and secreted
REV	Regulator of Virion
RNA	Ribonucleic acid
RT	Reverse Transcriptase
RTI	Reverse Transcriptase Inhibitor
SIV	Simian Immunodeficiency Virus
SIVcpz	Simian Immunodeficiency Virus of Chimpanzee
TAR	Transactivation response
TAT	Transactivator Protein
uL	micro liter
UN	United Nations
UNAIDS	The Joint United Nations Programme on HIV and AIDS
US	United States
VIF	Virion Infectivity Protein
VPR	Viral Protein R
VPU	Viral Protein U
VPX	Virion Protein
WHO	World Health Organisation

Amino Acids

A	Alanine
C	Cystein
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

ABSTRACT

Recently there has been increased access to ARVs in Uganda. There is high HIV diversity in Sub-Saharan Africa and very few data are available as to how subtype diversity may affect drug susceptibility and resistance. This study examined the genotypic HIV drug resistance in ART drug naïve patients in a Ugandan cohort as well as determining whether HIV-1 subtypes can be associated to drug resistance.

In a cross-section study of 104 ART naïve patients within one year of HIV seroconversion, 70 of the patients were genotyped for resistance to Reverse transcriptase inhibitors while 34 were genotyped for resistance to protease inhibitors. DNA was extracted from the PBMCs and both genes, the reverse transcriptase and Protease genes, were amplified by the PCR technique, after which they were then sequenced and analyzed for drug resistance using BioEdit sequence alignment editor (V 7.0.5.3) as well as the Stanford drug resistance data base. Subtyping was done using the Clustal X (V 1.83).

HIV-1 subtypes A and D were the two major ones found in the cohort. Prevalence of resistance to NRTIs was higher in subtype A patients (13.3%) than other subtypes (C and D), while resistance to NNRTIs was higher in subtype D patients (9.0%), than in C or D subtypes, though none of this was significant. Further more, resistance to reverse transcriptase inhibitors could not be associated to any of the subtypes.

All resistance mutations to protease inhibitors were minor drug mutations. Resistance to protease inhibitors was significantly higher in subtype A than D. Proportions of individuals that carried at least 2 drug resistance mutations for each of the subtypes A and D were 100% and 50% respectively.

There was a fairly high prevalence of resistance to reverse transcriptase inhibitors (12.9%). Though resistance to PIs was minor, this could lead to higher level resistance in presence of major mutations. Subtype A patients can therefore be predicted to fail PI therapy earlier than patients harboring subtype D virus because of the numerous minor resistance polymorphisms that can increase the fitness of the drug resistant virus.

CHAPTER ONE: **INTRODUCTION**

1.1 Background

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) have been identified as the primary cause of Acquired Immunodeficiency Syndrome (AIDS). HIV-1 is the major cause of AIDS globally today (Hoffman and Kamps, 2003; Grant and Cock, 2001). Antiretroviral drugs are medications for the treatment of infection by retroviruses, primarily HIV. Antiretroviral therapy has been available in Uganda since 1998, mainly provided by nongovernmental organizations, research studies and commercial providers (UNAIDS/WHO 2005). By the end of 2002, of the 600,000 people that were living with HIV infection in Uganda, 60,000 to 90,000 were in need of antiretroviral (ARV) therapy (Amolo *et al*, 2003). In 2004, the WHO estimated 114,000 people in Uganda were in need of ARV treatment and by the end of 2005, around 79,000 patients were on ARV treatment in Uganda. By the end of 2006, antiretroviral therapy (ART) was reaching 41% (96,000 patients) of the people in need of it (www.avert.org, 2007).

Though Uganda has been able to increase access to ART, some factors such as poor drug adherence or patient compliance, and irrational use and prescription of ARVs have led to development of drug resistance (Byakika *et al*, 2005). Resistance to antiretroviral drugs is one of the main reasons for treatment failure (Tobin and Frenkel, 2002). Exposure of the virus to antiretroviral drugs can contribute to development of drug resistant HIV strains, which can subsequently be passed on to drug naïve patients (Weinstock *et al*, 2004). Even with patients on Highly Active Antiretroviral Therapy (HAART), drug resistance has been shown to emerge. In one population study of patients who were on HAART, 72 to 80% of these patients had strains that were resistant to one or more antiretroviral drugs (Richman *et al*, 2004). Cross resistance can also emerge within a patient receiving antiretroviral therapy. This involves development of HIV strains resistant to antiretroviral agents of a particular class. Several studies have documented cross resistance. In their study, Delaugerre *et al*, 2001, showed that patients who were failing nevirapine then efavirenz therapy, developed cross resistance to all the available non-nucleoside reverse transcriptase inhibitors (NNRTIs). Increased clinical use of

combination antiretroviral treatment for HIV-1 infection has lead to the selection of viral strains resistant to multiple drugs including ones resistant to all the licensed, reverse transcriptase inhibitors (RTI) and protease inhibitors (Palmer *et al*, 1998).

1.2 Problem statement

As more people access antiretroviral therapy, HIV drug resistance becomes a major challenge. The pattern and level of drug resistance is particularly not clear in non-B subtypes. HIV1 strains harboring drug resistance mutations have been isolated from treated and untreated patients (Richards *et al*, 2004). Development of HIV drug resistant strains is one of the main reasons for failure of antiretroviral therapy. This limits the number of alternative regimen and the virological success of salvage regimens (Hoffman and Kamps, 2003). The salvage regimens are usually more expensive to acquire both to the patient and to the country or the institution funding the purchase of these drugs. Cross-resistance may affect all currently available anti-HIV drugs to a greater or lesser extent. Also for some drugs, especially the non-nucleoside reverse transcriptase inhibitors (NNRTI), cross resistance may set in early. This affects the choice of another drug in the same drug class.

1.3 Objectives

1. 3.1 General Objective

To determine the genotypic HIV drug resistance in HIV/AIDS` patients naïve to drugs in Uganda.

1.3.2 Specific aims

- I. To determine the prevalence of genotypic HIV drug resistance in ARV naïve patients.
- II. To determine the genotypic HIV drug resistance in each of the subtypes A and D in Uganda
- III. To determine the prevalence of transmitted HIV drug resistance
- IV. To compare the prevalence of HIV drug resistance in the subtypes A and D so as to determine whether subtypes are associated to drug resistance.

1.4 Justification

Most studies on HIV drug resistance have been done on the B subtype and yet some new evidence has shown HIV drug resistance mutations that are unique to non B subtypes. Gao *et al*, 2004 characterized a high level of pre-treatment resistance mutations that were unique to the D subtype of HIV-1. A V106M mutation in HIV-1 unique to clade C viruses exposed to efavirenz has been shown to confer cross-resistance to non-nucleoside reverse transcriptase inhibitors (Turner *et al* 2003). Therefore this necessitates more studies to be done on the non B subtypes to further determine the pattern of drug resistance in these subtypes and help guide therapy.

It's inevitable, resistant strains will always emerge (even with HAART) because of the high rate of HIV mutation. Therefore there is need for regular monitoring of HIV drug resistance to control further emergence of drug resistance strains of HIV-1.

Much as ART has been beneficial in the management of HIV/AIDS, HIV-1 has been able to evolve strains that are resistant to the ARVs. Very few data are available as to how subtype diversity may affect drug susceptibility and resistance (Weinberg *et al*, 2004). Some studies have tried to link emergence of HIV drug resistance to subtypes. Richards *et al*, 2004 showed that HIV drug resistance was higher in subtype D than in A. Baker *et al*, 2007 showed a high rate of NNRTI polymorphism in the D subtype than A while the Nucleoside reverse transcriptase inhibitor (NRTIs) polymorphism was higher in the A subtype than D. Results are not conclusive. Therefore there is need to find out whether there is an association between subtypes and the prevalence of HIV drug resistance.

1.5 Significance

Establishment of the current level and pattern of HIV drug resistance will enable clinicians make informed decisions regarding ARV drug regimen choices for HIV patients especially in settings without resistance testing. This will lead to better choice of regimen, treatment outcome and quality of life. Knowledge of drug resistance patterns will assist in designing empiric regimen since it is difficult to obtain prior knowledge of the drug resistance profile of the infecting virus.

1.6 Research question

What is the current drug resistance profile of HIV-1 in patients in Uganda?

1.7 Hypothesis

The null Hypothesis: Resistance is independent of subtypes.

CHAPTER TWO: LITERATURE REVIEW

2.1 HIV/AIDS epidemiology

Human Immunodeficiency Virus type 1 (HIV-1) and type 2 (HIV-2) have been identified as the primary cause of Acquired Immunodeficiency Syndrome (AIDS). HIV-1 is the major cause of AIDS globally today (Hoffman and Kamps, 2003; Grant and Cock, 2001). HIV-2 is mainly found in West Africa. Individual cases of HIV-2 infection have been described in other parts of Africa, Europe, America, and Asia (India), but most people with HIV-2 infection have some epidemiological link to West Africa (Grant and Cock, 2001). Worldwide, more than 40 million people are infected with HIV (Hoffman and Kamps, 2003). Ninety-five percent of these cases are in the developing countries, generally in sub-Saharan Africa and Southeast Asia. Since the AIDS epidemic began, more than 20 million deaths have been attributed to AIDS (Dubin, 2004).

Uganda is estimated to have a population of about 25-30 million. The U.S. Census Bureau/Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated the national HIV prevalence to have fallen to 5 percent as of 2001. By the end of 2003, the government and the UN estimated 4.1% of adults had HIV-1 infection. Uganda's Ministry of Health estimated the adult prevalence of HIV/AIDS (15-49 years) in 2005 to be 6.4% (Uganda HIV/AIDS Sero-Behavioural Survey, May 2005).

2.2 HIV subtypes; phylogeny and distribution

HIV is a Lentivirus, a subgroup of retroviruses. This family of viruses is known for latency, persistent viremia, infection of the nervous system, and weak host immune responses (Dubin, 2004). HIV-1 is classified into three groups which include (fig 1): The major group which is M, Outlier group O and the new group N. More than 90% of HIV-1 infections are caused by group M. Within group M there are nine genetically distinct subtypes or clades. These are A, B, C, D, F, G, H, J and K. Some subtypes can form hybrid virus and those hybrid viruses which are able to infect more than one person are known as circulating recombinant forms or CRFs. CRF A/B is a mixture of subtype A and B (Nobel, 2004). Subtype E has never been purified, and is always seen combined with subtype A as CRF A/E (Goudsmit, 1997). Subtypes B and C are the most widely spread. B is mainly found in Europe, America, Japan and Australia. Subtype C is

predominant in southern and eastern Africa, India and Nepal. It is responsible for half of the World's HIV infections. Subtype A is found in west and Central Africa and also causing much of the epidemic in Russia. Subtype D is generally limited to east and central Africa; A/E recombinant is prevalent in south-east Asia, but originated in central Africa; F has been found in central Africa, South America and Eastern Europe; G and A/G have been observed in western and eastern Africa and central Europe. Subtype H has only been found in central Africa; J only in Central America; and K only in the Democratic Republic of Congo and Cameroon. A and D are the major subtypes in Uganda (Nobel, 2004).

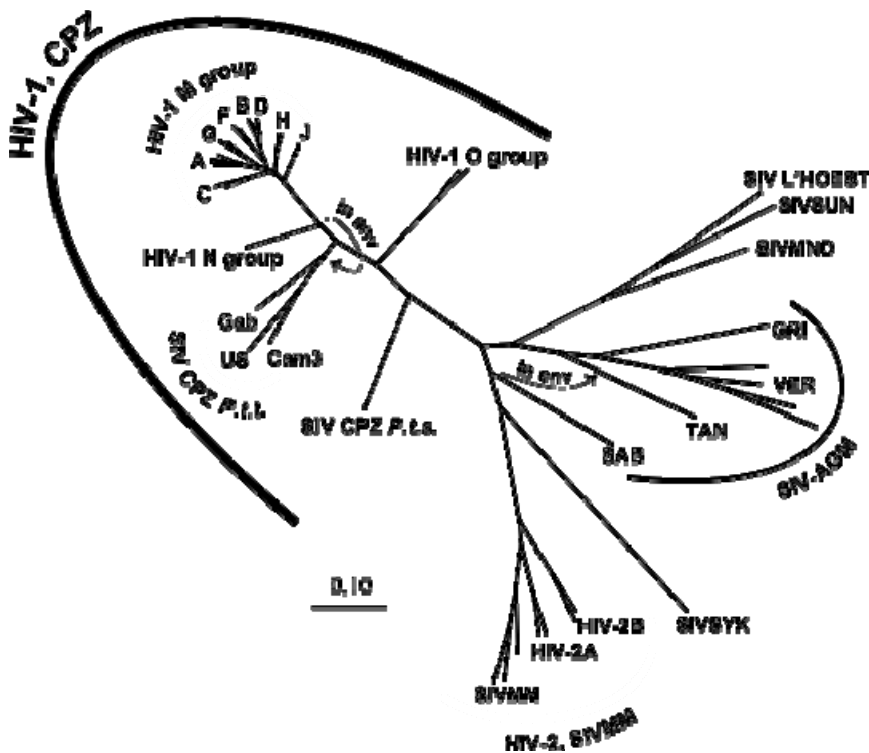


Fig 1 Phylogenetic tree of HIV and SIV. SIV is closely related to HIV and the origin of HIV is generally attributed to SIV. HIV-1 is closely related to the chimpanzee strain of SIV, designated SIVcpz. HIV-2 is most closely related to SIVsm, the SIV strain that primarily infects Sooty Mangabeys. (Figure adapted from Wikipedia)

2.3 Methods of HIV/AIDS control

HIV can be transmitted in three main ways: Sexual transmission, transmission through blood and Mother-to-child transmission, which is the infection of HIV from an HIV-positive mother to her child during pregnancy, labour, delivery or during breastfeeding (www.avert.org). Most methods of control of HIV/AIDS are aimed at preventing spread through the above ways of transmission.

Sexual transmission can be controlled by any of the following methods: Abstaining from sex or delaying first sex, being faithful to one partner or having fewer partners and condom use, which means using male or female condoms consistently and correctly ([www. Avert.org](http://www.Avert.org), 2004). Education programs to teach people on safer sexual behavior such as abstaining or delaying sex until marriage, being faithful to one partner, proper use of condoms have been conducted. These have targeted the youth, school children, and other kind of people at risk. Numerous studies have shown that condoms, if used consistently and correctly, are highly effective at preventing HIV infection (www.avert.org, 2005). Another significant intervention is providing treatment for sexually transmitted infections, such as chlamydia and gonorrhoea. This is because such infections, if left untreated, have been found to facilitate HIV transmission during sex (www.Avert.org, 2007). Combination prevention refers to strategies to prevent sexual transmission of HIV. The “A, B, Cs” of combination prevention include; Abstinence, being safer (by being faithful or reducing the number of partners), and correct and consistent condom use. A, B, and C interventions can be adapted and combined in a balanced approach that will vary depending on cultural context, the population targeted and the stage of the epidemic (UNAIDS; Facts Sheet, 2005).

Effective prevention of mother-to-child transmission (PMTCT) requires a four-fold strategy (Duerr et al, 2005); (i) Preventing HIV infection among prospective parents (ii) Avoiding unwanted pregnancies among HIV positive women (iii) Preventing the transmission of HIV from HIV positive mothers to their infants during pregnancy, labour, delivery and breastfeeding (iv) Use of antiretroviral drugs, safer feeding practices and other interventions. For HIV-infected pregnant women whose condition does not require

ART or where ART is not available, the WHO recommends antiretroviral prophylaxis for PMTCT whereby AZT is started at 28 weeks of gestation, intrapartum SDNVP, and single-dose infant NVP combined with 1 week of AZT. In addition, intrapartum AZT/3TC followed by 7 days of maternal postpartum AZT/3TC is recommended to reduce the development of NVP resistance (Halima et al, 2006).

2.4 Prevalence of HIV drug resistance

Development of HIV drug resistance is one of the major reasons for treatment failure (Wegner *et al* 2000). Prevalence of HIV drug resistance is a dynamic phenomenon. Whenever there is a change in the use of ART, there is expected to be a change in the prevalence of HIV drug resistance which may increase when there is increased ARV use and which may decrease when there is decreased use. In regions where there is wide spread use of ART, HIV drug resistance is higher than in areas where ART has just become available. Booth *et al*, 2007, showed that ART drug naïve people that were born in the United Kingdom had a higher prevalence of resistance to ARVs than those not born in the United Kingdom. Homosexual males, persons with subtype B and those born in the United Kingdom showed the highest rates of resistance. Multivariate analysis indicated that having been born in the United Kingdom was the key predictor of resistance. In Tanzania where access to ART is just being scaled up, HIV drug resistance was categorized as being less than 5%, which is much lower than in countries where ART has been available for a much longer time. Also before treatment the prevalence of HIV drug resistance appears to be lower than in ART experienced patients (Pillay *et al*, 2007, Richards *et al*, 2004). In one study among ART naïve patients from Greece the overall prevalence of ARV drug resistance was 9% and resistance to NRTIs and NNRTIs was 5% and 4% respectively. There was no resistance to PIs (Paraskevis *et al*, 2005).

Some of the studies done in Uganda before ART had become widely available found a higher prevalence of resistance, which was 52% in the ART treated group (Richards *et al*, 2004). The majority of these mutations were found in the reverse transcriptase gene. The reason for this prevalence could be due to the suboptimal drug regimens that fail to adequately suppress viral replication resulting into selection of resistant viral quasi species. Most of these people were practicing a form of treatment interruption due to the

prohibitive treatment costs at that time. Many other studies have looked at patients naïve to ARVs in Uganda. Pillay *et al*, 2007 studied patients before starting ART, and at 24 and 48 weeks after ART initiation. There was a prevalence of 10% among the drug naïve samples. For these patients resistance to NRTIs, NNRTIs and PIs was 6%, 4% and 3% respectively. Resistance to reverse transcriptase inhibitors could be explained by the fact that these drugs are commonly used in Uganda and could partly be due to transmitted resistance or prior therapy to these drugs. In this same study it was also revealed that at 48 weeks, the prevalence of resistance was higher than that at baseline (before ART initiation) supporting the fact that resistance will always increase whenever there is use of ART.

As prevalence of resistance to ART increases, there is also an increased probability of resistant virus being transmitted (Wainberg and Friedland, 1998). Though some mutations compromise the replication fitness of the virus (Briones *et al*, 2001) transmitted resistant strains appear to have a higher replication capacity that enables it to dominate over the wild type virus (Simon *et al*, 2003). Also transmitted resistant viruses can partially revert to wild type virus resulting into mixtures of wild type and resistant virus (Little *et al*, 2004). Failing ART results into increased blood HIV RNA levels and the viruses are usually resistant to the drugs which the patient is taking (Quinn *et al*, 2000). This then leads to increased potential for transmission of HIV, including drug-resistant strains. Poor drug adherence and suboptimal ARV drug regimen do contribute to drug resistant HIV which can then be transmitted. Most studies report that at least 10% of new primary HIV-1 infected people carry virus resistant to at least one of the antiretroviral drugs while they are still therapy naïve, suggesting that they have been infected with drug resistant virus (Palanee *et al*, 2003). Some studies done in Uganda have reported a prevalence of 10% (Pillay *et al*, 2007).

2.5 Structure of HIV genome and functions of the different proteins

HIV is a retrovirus composed of two copies of single stranded RNA that are enclosed within a nucleocapsid and the HIV genome is flanked by a repeated sequence called Long Terminal Repeat (LTR) region. The genome of HIV is composed of several genes (Fig. 2) that encode at least nine different proteins. These proteins are grouped into three groups, each group playing its own major role. The *gag*, *pol*, and *env* genes code for their respective proteins, Gag, Pol, and Env, which are the major structural proteins. The regulatory proteins being the Tat and Rev. The accessory proteins include; Vpu, Vpr, Vif and Nef. The functions of these proteins are described below.

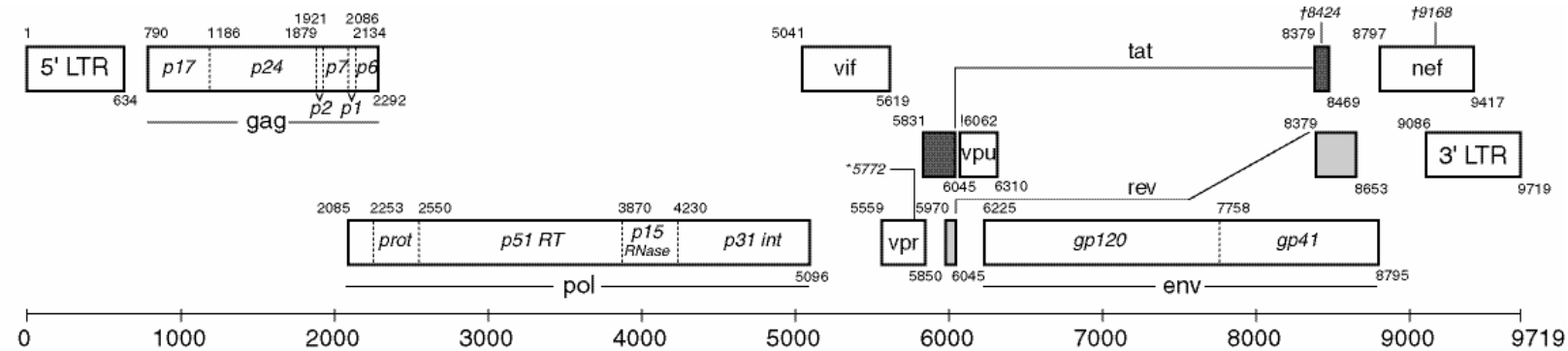


Fig 2 Linear structure of HIV genome. Adapted from the Los Alamos HIV sequence database.

Structure of the HIV genome is about 9700 base pairs long. The three major genes, *gag*, *pol*, and *env* code for their different respective polyproteins which are cleaved to give rise to mature HIV functional proteins. Most of the currently available antiretroviral drugs target and inhibit the functions of these proteins particularly in the *pol* and *env* regions of HIV genome. For instance the protease inhibitors target and inhibit the protease enzyme from the protease gene, Reverse transcriptase inhibitors target the reverse transcriptase enzyme. Integrase inhibitors which are a new and most recent class of ARV drugs targets the integrase enzyme of HIV preventing the incorporation of proviral DNA into the host cell genome. Fusion inhibitors prevent the fusion of viral membrane proteins and the host cell membranes, thereby denying entry of HIV into the host cell. Most of the other proteins of HIV, such as the *vif*, *vpr*, *vpu*, *nef*, remain as potential antiretroviral targets that need to be / are being investigated because of their role in HIV replication and infectivity.

2.4.1 The *gag* group-specific Antigen) gene: This codes for a 55kd Gag precursor unspliced protein called the p55. During its translation, the p55 N terminus undergoes myristoylation, thereby enabling its association with the host cell's cytoplasmic membranes followed by uptake of two copies of viral genomic RNA. This then triggers budding off of the viral particle from the infected cell. After budding, p55 is cleaved by the viral protease during the process of viral maturation into four smaller proteins ; the matrix protein, MA (p17]), capsid protein, CA (p24), nucleocapsid proteins, NC, p6 and p7 (Gottlinger, *et al*, 1989).

2.4.2 The MA polypeptide is derived from the N-terminal, myristoylated end of p55. The MA stabilizes the virion particle and it's involved in the transport of the viral genome into the nucleus. It carries a karyophilic signal which is recognized by the cellular nuclear import machinery, thereby enabling the transport of the viral particle into the host cell nucleus.

2.4.3 The p24 (CA) protein forms the conical core of viral particles. p24 protein together with gp 120/160 and gp 41, are the proteins detected by the western blot test for HIV.

2.4.4 The NC (p7) recognizes and binds to the HIV packaging signal through interactions mediated by two zinc-finger motifs (Harrison *et al*, 1992, Poznansky *et al*, 1991). It also facilitates reverse transcription (Lapadat *et al*,1993). Generally the NC has many roles during the viral life cycle, such as placement of the tRNA primer, the maturation of the dimeric RNA genome into a more compact and thermostable form, and the minus- and plus-strand transfer during reverse transcription (Rein *et al*,1998).

2.4.5 The p6 is the only *gag* domain that is absent from the Gag-pol polyprotein and is the most variable domain in terms of length and sequence among the Gag proteins (Göttlinger 2001). The p6 protein is involved in the release of virions from the host cell. The p6 polypeptide region also mediates interactions between p55 Gag and the accessory protein Vpr, leading to the incorporation of Vpr into assembling virions(Paxton *et al*, 1993).

2.4.6 The Pol: The viral enzymes, Protease, Integrase, RNase H and Reverse transcriptase are produced as a Gag-Pol precursor protein that is cleaved into these proteins by the viral protease. The Gag-Pol precursor (p160) is generated by a ribosomal frame shifting near the 3' end of *gag*., which is triggered by a specific cis-acting RNA motif (Parkin *et al*, 1992). The HIV-1 protease is an aspartyl protease enzyme that cleaves Gag and Gag-Pol polyprotein precursors during virion maturation into their respective separate functional proteins (Davies, 1990). It cleaves at Phe-Pro sites in Gag and Gag-Pol polyproteins. Without effective HIV PR, HIV virions remain uninfected.

2.4.6.1 The HIV protease enzyme is a homo dimer, with each subunit made up of 99 amino acids. The active site lies between the two subunits with a characteristic amino acid sequence common to aspartic proteases (Davies, 1990). The protease enzyme is the protein targeted by protease inhibitor drugs to inhibit or suppress the replication of HIV.

The pol reverse transcriptase is an RNA-dependent and DNA-dependent polymerase that makes a double-stranded DNA copy of the dimer of single-stranded genomic RNA present in the virion. It has both the synthetic polymerase activity and the RNase H that removes the original RNA template from the first DNA strand, allowing synthesis of the complementary strand of DNA (Hope and Didier, 2000). The predominant functional species of the polymerase is a heterodimer of p65 and p50. All of the *pol* gene products can be found within the capsid of free HIV-1 virions. Because the polymerase does not contain a proof-reading activity, replication is error-prone and introduces several point mutations into each new copy of the viral genome.

2.4.6.2 Integrase: The integrase enzyme mediates the insertion of the proviral DNA into the host cell's genome (Bushman *et al*, 1990). It has the exonuclease activity that trims two nucleotides from each 3' end of the linear viral DNA duplex, an endonuclease activity that cleaves the host DNA at integration sites, and the ligase activity that generates a single covalent linkage at each end of the proviral DNA. Rather than specific sequences within the host cell DNA, the accessibility of the DNA within the chromatin is the choice for integration of viral DNA into host DNA (Pryciak and Varmus, 1992).

2.4.7 The envelop: The *envelop* gene (*env*) codes for the precursor glycoprotein 160 (gp160), which is produced from singly spliced mRNA. The gp 160 is processed to give a noncovalent complex of the external glycoprotein gp120 and the transmembrane glycoprotein gp41 by cellular proteases. gp120 is located on the surface of the infected cell and of the virion through noncovalent interactions with gp41. Env exists as a trimer on the surface of infected cells and virions (Bernstein *et al*, 1995). The binding site for the CD4 receptor, and the seven transmembrane domain chemokine receptors that serve as co-receptors for HIV-1 are found on gp120. Gp120 has five hypervariable regions, designated V1 through V5 loops. The gp41 moiety contains an N-terminal fusogenic domain that mediates the fusion of the viral and cellular membranes, the virions inner components are then transferred into the cytoplasm of the newly infected host cell (Camerini and Seed1990).

2.4.8.0 The regulatory proteins: These are two, TAT and Rev.

2.4.8.1 TAT: Is the transcriptional transactivator that primarily controls HIV transcription. Kao *et al.*, 1987, showed that cells transfected with HIV long terminal repeats LTR predominantly produced short non-polyadenylated RNAs that terminate near the promoter in absence of Tat whereas in the presence of Tat the long transcripts predominated. Tat acts principally to promote the elongation phase of HIV-1 transcription. Tat binds to the transactivation response (TAR) RNA element activating transcription initiation and elongation from the LTR promoter, preventing the 5' LTR AATAAA polyadenylation signal from causing premature termination of transcription and polyadenylation (Kao *et al*,1987). Two forms are known, Tat-1 exon (minor form) of 72 amino acids and Tat-2 exon (major form) of 86 amino acids. These are expressed by early fully spliced mRNAs or late incompletely spliced HIV mRNAs, respectively. Both forms function as transcriptional activators and are found within the nuclei and nucleoli of infected cells.

2.4.8.2 The REV protein : Found localized primarily in the nucleolus and nucleus of infected cells. Rev binds to the Rev response element (RRE), that lies within the second

intron of HIV, thereby facilitating the export of unspliced and incompletely spliced viral RNAs from the nucleus to the cytoplasm. Rev cycles rapidly between the nucleus and the cytoplasm. Higher levels of Rev expression can lead to increased transfer of RNAs that contain introns, much more than is supposed to be transferred to the cytoplasm from the nucleus. This in turn leads to inhibition of Rev expression (Felber *et al.*, 1990).

2.4.9.0 The Accessory proteins: Nef, vif, vpr, vpu (in HIV-1 and SIVCPZ), and vpx (in HIV-2 and other SIV strains) are the accessory proteins in lentiviruses. They are responsible for different important virulence factors. They are not absolutely required for viral replication in all in vitro systems (Cullen, 1998)).

2.4.9.1 The NEF protein: This is a 27-kD myristoylated protein of 206 amino acids that associates with cellular membranes (Niederman *et al.*, 1993) and is encoded by a single exon that extends into the 3' LTR. Nef is abundantly produced during the early phase of viral gene expression (Guy *et al.*, 1987; Klotman *et al.*, 1991) and some of its functions include the down regulation of the cell surface expression of CD4 and MHC class I, (Garcia and Miller, 1991; Guy *et al.*, 1987), the stimulation of virion infectivity (Lama *et al.*, 1999;), and the capacity to alter the activation state of infected cells. CD4 down regulation is beneficial to viral production because an expression of CD4 on the cell surface has been found to inhibit virion budding and Env incorporation into the virion thereby lowering HIV infectivity. In single-round infectivity assays, Nef-defective viruses produced from CD4-negative cells are from three to ten times less infectious than those from viruses with the Nef gene. Additionally, Nef-mutated virions show a severe growth defect in primary blood lymphocytes infected while resting and subsequently activated (Miller *et al.*, 1994; Spina *et al.*, 1994). Virions produced in the absence of Nef are less efficient in reverse transcription, although Nef does not appear to affect directly the process of reverse transcription (Schwartz *et al.*, 1995).

2.4.9.2 The Vpr (viral protein R) is a 96-amino acid (14-kD) protein. It interacts with the p6 Gag part of the Pr55 Gag precursor. VPR mediates the nuclear import of preintegration complexes from the cytoplasm thereby enabling HIV infection of non

dividing cells (Heinzinger, 1994), blocks cell division, transactivation of cellular genes, and induction of cellular differentiation (Rogel *et al*, 1995).

2.4.9.3 The Vpu (viral protein U) is unique to HIV-1, SIVcpz (the closest SIV relative of HIV-1), SIV-GSN, SIV-MUS, SIV-MON and SIV-DEN. The 16-kD Vpu polypeptide is an integral membrane phosphoprotein that is primarily localized in the internal membranes of the cell. Vpu is expressed from the mRNA that also encodes env (Sato *et al*, 1990) the two functions of VPU include degradation of CD4 in the endoplasmic reticulum, and enhancement of virion release from the plasma membrane of HIV-1-infected cells (Schwartz *et al*,1990).

2.4.9.4 The Vif is a 23-kD polypeptide that is essential for the replication but not production of viral particles in peripheral blood lymphocytes, macrophages, and certain cell lines. Vif prevents the encapsidation of APOBEC3G and APOBEC3F, the two potent antiretroviral cytidine deaminases which deaminate DNA:RNA heteroduplexes in the cytoplasm (Qin *et al*,2003).

2.4.9.5 The Vpx : A virion protein of 12 kD found in HIV-2, SIV-SMM, SIV-RCM, SIV-MND-2, and SIV-DRL and not in HIV-1 or other SIVs. This accessory gene is a homolog of HIV-1 vpr, and viruses with vpx carry both vpr and vpx.

2.6 HIV life cycle

Binding and Viral entry: Viral entry involves fusion of the viral envelope with the host cell membrane. Two viral envelope proteins, gp120 and gp41, and two host cell receptors (CD4 and the co-receptors) are involved (Doms and Trono, 2000). The two viral envelope proteins, gp120 and gp41, are conformationally associated to form a trimeric functional unit consisting of three molecules of gp120 exposed on the virion surface and associated with three molecules of gp41 inserted into the viral lipid membrane. Binding of the HIV env receptor, trimeric gp120, to the host cell CD4 receptor molecule is the initial stage of infection (Kwong *et al*, 1998). This then results into a conformational change in gp41 which contains heptad repeats (HR-1 and HR-2) (Hunter, 1997). This conformation leads

to the binding of the gp41 to one of the co-receptors, usually the chemokine receptor CXCR4 in the case of T-cell-tropic, or syncytium-inducing strains of HIV or the chemokine receptor CCR5 in the case of macrophage-tropic, or nonsyncytium-inducing strains (Scarlati *et al*, 1997).

Binding to co-receptors causes the formation of the hairpin intermediate that pulls the virus and the host cell membranes together thereby enabling fusion to occur (Hunter, 1997). HIV virions can also enter cells by endocytosis though this does not result into infection possibly because of inactivation by endosomes. However, a special form of endocytosis has been demonstrated in submucosal dendritic cells. These cells have a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells (Geijtenbeek *et al*, 2000). This C-type lectin binds HIV gp120 with high affinity but does not trigger the conformational changes required for fusion. Instead, virions bound to dendritic cell specific C type lectin (DC-SIGN) are internalized into an acidic compartment and subsequently displayed on the cell surface after the dendritic cell has matured and migrated to regional lymph nodes, where it engages T cells (Kwon *et al*, 2002).

Uncoating: Once inside the cell, the virion remains attached to the cell membrane as it undergoes uncoating. A number of processes that enable successful uncoating of the virus seem to take place; phosphorylation of viral matrix proteins by a mitogen-activated protein (MAP) kinase (Cartier *et al*, 1999), Viral protein Nef enhances cytoplasmic delivery of virions by inducing local changes in pH, through the universal ATPase pump (Lu *et al*, 1998). Nef also down regulates the cell surface expression of CD4 and MHC class I (Garcia and Miller, 1991). Viral Vif protein stabilizes the reverse transcription complex by preventing the encapsidation of APOBEC3G and APOBEC3F. These antiretroviral cytidine deaminases deaminate DNA:RNA heteroduplexes in the cytoplasm (Qin *et al*, 2003) thereby preventing HIV replication.

Reverse transcription: After uncoating, viral contents are then transported into the cytoplasm of the host cell. Among these is the viral reverse transcription complex (Karageorgos *et al*, 1999). This complex, among others includes a diploid viral RNA

genome, lysine transfer RNA which acts as a primer for reverse transcription, viral reverse transcriptase enzyme that catalyses the synthesis of proviral DNA using viral RNA as a template, integrase enzyme that catalyses the insertion of the proviral DNA into the host cell's genome (Bushman *et al*, 1990), matrix and nucleocapsid proteins, and viral protein R (Vpr) whose many functions include: blocking cell division, transactivation of cellular genes, and induction of cellular differentiation (Rogel *et al*, 1995). Once inside the host cell, reverse transcription takes place giving rise to the pre integration complex (PIC). PICs are large nucleoprotein particles that carry out proviral DNA integration into the host cell chromosome. The PIC is composed of a double-stranded proviral DNA, integrase, matrix, Vpr, reverse transcriptase, and the high mobility group DNA-binding cellular protein HMGI (Y) of the host cells (Miller *et al*, 1997). Within the PIC, the proviral DNA is condensed in a partially disassembled remnant of the viral core, with proteins tightly associated at the apposed proviral DNA ends but loosely associated with the intervening proviral DNA.

The integrase enzyme has an endonuclease activity that cleaves the proviral DNA. This creates a defined end at the heterogeneous cDNA termini (Miller *et al*, 1997 and Nair *et al*, 2007). This 3' processing is important for producing molecules that are capable of being integrated into the host genome. The 3' processing reaction removes 2 nucleotides from the 3' end of the DNA molecule so that it mimics either the U3 or U5 end of HIV DNA. The PIC then has to enter the host cell nucleus, where the viral enzyme integrase mediates the insertion of the proviral DNA into the host chromosomal DNA.

Import of the PIC into the nucleus: A number of viral factors that are present in HIV-1 preintegration complexes (PICs) have been thought to be involved in nuclear import of the PIC itself. HIV VPr has been described as a nucleocytoplasmic shuttling protein. VPr has at least two distinct nuclear import signals that enable it to bypass the classical importing alpha/beta-dependent signals without the requirement for energy (Sherman *et al*, 2001). Thus among other functions VPr protein may carry the signal for nuclear import of the PIC.

Integration: Following the 3' processing reaction which yields integratable ends of HIV DNA, is the strand transfer reaction, which occurs in the host cell nucleus (Miller *et al*, 1997). This reaction inserts a 3' end processed viral DNA into the target or host DNA so that the 3' end of the viral DNA is joined to the 5' end of the target DNA at the site of insertion. There is very little target site specificity and as such any DNA can serve as target DNA including the viral DNA itself (Karn, 1995).

The proviral DNA is then transcribed with the help of the host cell factors as well as some of the viral proteins such as Tat and Rev (Wei *et al*, 1998). The integrated viral DNA carries duplicated structures called the Long Terminal Repeats (LTR) at each end. The 5' LTR functions as a promoter element while the 3' LTR supplies the polyadenylation signal (Karn, 1995). Tat stimulates transcription from the viral LTR. Tat acts principally to promote the elongation phase of HIV-1 transcription (Kao *et al*, 1987). Rev is required for efficient expression of viral mRNA encoding the structural proteins of the virus (Goh *et al*, 1998 and Karn, 1995).

The viral RNA is transported out of the nucleus into the cytoplasm either as a full-length HIV genome to be packaged as genetic material for new virions, or it may be partially or fully spliced. The unspliced, partially spliced, and fully spliced versions of viral RNA direct the synthesis of different viral proteins by the cell ribosomes. During assembly, Gag associates with cholesterol-enriched microdomains or rafts at the plasma membrane. This association of Gag with plasma membrane rafts is an important step in HIV-1 replication (Ono and Freed, 2001). The new viral particles are assembled at the plasma membrane and incorporate Gag subunits, Pol, Nef, Env, Vpr, and viral genome. The HIV viral protease enzyme acts following virion assembly to cleave viral proteins into functional structural and enzymatic components. The Nef protein protects the infected cells against the killing by the cytotoxic T lymphocytes (Collins *et al*, 1998)

2.7 Antiretroviral drugs: Mechanisms of Action.

Antiretroviral drugs are medications for the treatment of infection by retroviruses, primarily HIV (www.wikipedia.org/wiki/Retrovirus).

Currently there are five classes of these agents: nucleoside and nucleotide analogs (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), the entry inhibitors and the integrase inhibitors. These drugs inhibit the different stages of HIV-1 replication cycle. Highly Active Antiretroviral Therapy (HAART) is the recommended treatment for HIV infection. HAART combines three or more anti-HIV medications in a daily regimen. Anti-HIV medications do not cure HIV infection and individuals taking these medications can still transmit HIV to others. Both NRTIs and NNRTIs are reverse transcriptase inhibitors. Their target is the reverse transcriptase enzyme (Hoffman and Kamps, 2003).

Nucleoside analogues such as zidovudine, stavudine and lamuvidine resemble the endogenous nucleoside triphosphates, (fig 3). They are converted to the active metabolites only after endocytosis, where by they are phosphorylated to triphosphate derivatives (Hoffman and Kamps, 2003). After the activation of these analogues, the resultant 5'triphosphates then compete with endogenous nucleoside triphosphates for incorporation into proviral DNA (Hoggard, 2000). The incorporation of these analogues aborts DNA synthesis, as phosphodiester bridges can no longer be built to stabilize the double strand (Hoffman and Kamps, 2003).

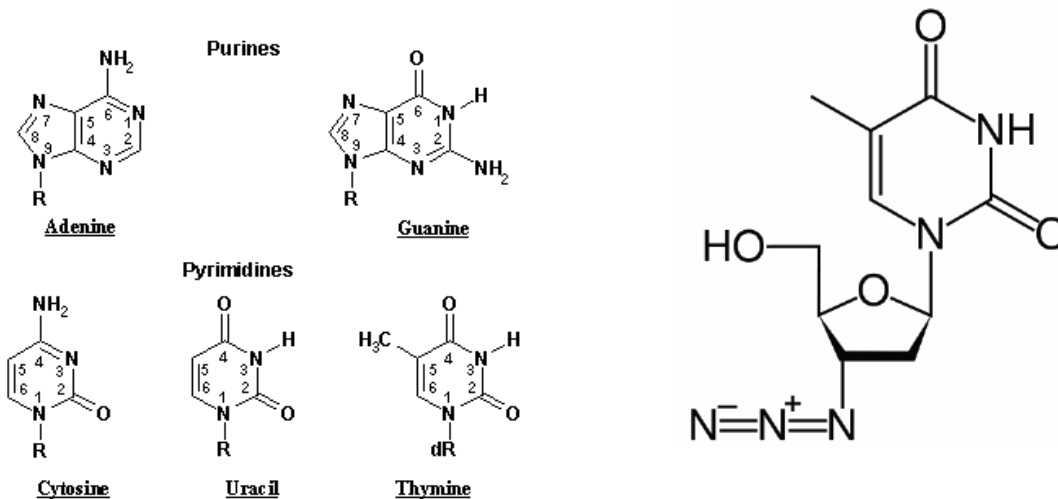


Fig 3: Endogenous nucleoside triphosphates (left) and structure of zidovudine (AZT) on the extreme left, one of the NRTIs. Uracil is only found in RNA while Thymine is found only in DNA. The rest of the three

bases are found in both DNA and RNA. NRTIs drugs are analogues to any of the above triphosphates. NRTIs compete with endogenous nucleoside triphosphates for incorporation into proviral DNA.

Delavirdine, nevirapine, efavirenz and etravirine are the only currently approved NNRTIs (fig 4). Unlike the NRTIs, NNRTIs are not “false” building blocks (Hoffman and Kamps, 2003) and do not require intracellular phosphorylation to become active to inhibit HIV-1 (Zapor *et al* 2004). NNRTIs bind directly and non competitively to the reverse transcriptase enzyme at a position in close proximity to the substrate binding site for nucleosides (Hoffman and Kamps, 2003). This results into a conformational change at the active site of the enzyme decreasing its affinity for nucleoside binding. This significantly decreases DNA polymerization and hence the number of viral particles produced.

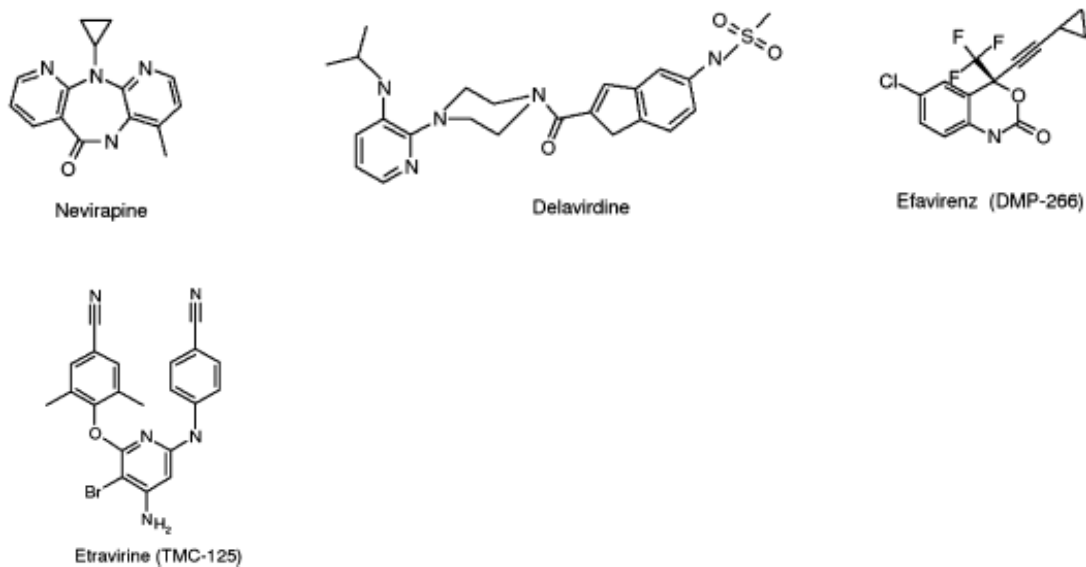
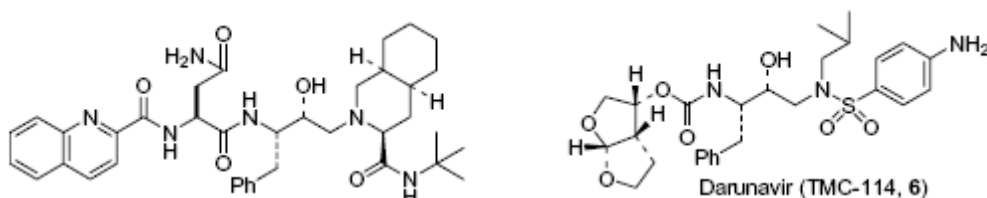


Fig 4: NNRTIs non-competitively inhibit the reverse transcriptase enzyme. Etravirine is the most recently FDA approved NNRTI. It is effective against HIV strains that are resistant to the earlier NNRTIs above.

The HIV protease enzyme cuts the viral Gag-Pol polyprotein into its functional subunits. Inactivation of HIV-1 protease by either mutation or chemical inhibition leads

to the production of immature, noninfectious viral particles (McQuade, 1990). Most of the inhibitors cocrystallised with HIV protease are bound in the enzyme active site in an extended conformation (Wlodawer and Vondrasek, 1998). Protease inhibitors can have an alternative target which is the dimer interface. Inhibitor binding introduces substantial conformation changes to the enzyme (Wlodawer and Vondrasek, 1998). This prevents the actual substrates from binding to the enzyme. Some of the protease inhibitors include Saquinavir, Nelfinavir, Lopinavir, Tipranavir and Darunavir (fig 5).



Saquinavir.

Fig 5: Structures of two Protease Inhibitor drugs. Saquinavir, one of the earliest PIs and darunavir which is the most recent PI. Darunavir was designed to combat HIV drug resistant strains.

The binding of gp120 to CD4 is a crucial step for viral entry. HIV needs to bind one of the chemokine receptors CCR5 and CXCR4 to actually infect its target cells (CCR5 CXCR4 CD4 positive cells). The ectodomain of gp41 contains a hydrophobic fusion peptide sequence at the amino terminus, and heptad repeats (HR-1 and HR-2) (Hunter, 1997). After binding of gp120 to CD4, a conformation change is also induced in gp41. This leads to the formation of an HR-1 coiled coil called prehairpin intermediate. The intermediate forms a stable trimer of hairpins called six-helix bundle after interaction with the HR-2 (hydrophobic heptad repeat of gp41) The peptide fusion inhibitors like Enfuvirtide prevent the transition from the prehairpin intermediate to the fusion-active six-helix bundle by competitively binding to targets on the HR-1 coiled-coil structure. This prevents interactions between the HR1 and HR2.

The beta chemokines MIP-1alpha (macrophage inflammatory protein 1 α), MIP-1beta and RANTES (regulated upon activation, normal T-cell expressed and secreted) inhibit CCR5-dependent membrane fusion mediated by the envelope glycoprotein of HIV-1 (Ghalib Alkhatib *et al.*, 1997; Dragic *et al.*, 1996; Cocchi *et al.*, 1996). The chemokine receptor CCR5 is a necessary co-receptor for monocytotropic (M-tropic) HIV 1 isolates while CXCR4 is used by T-cell tropic HIV-1 isolates. SDF-1 (stromal cell derived factor 1) is a natural ligand for CXCR4 and is able to inhibit the entry of T tropic HIV 1 isolates into activated CD4 T cells. RANTES, MIP-1 α , and MIP-1 β are the natural ligands for CCR5 and are able to inhibit entry of M-tropic HIV-1 isolates into T cells (Hoffman and Kamps, 2003). The chemically modified chemokines inhibit HIV-1 infection more efficiently than the natural ligands. These N-terminally modified RANTES include: RANTES 9-68, Met-RANTE, AOP-, NNY-, and PSC-RANTES. Their mechanisms of action are said to be by receptor blockade and receptor sequestration (Pastore *et al.*, 2003). Maraviroc is a recently FDA approved CCR5 inhibitor.

Integrase inhibitors are a new class of antiretrovirals. They inhibit the integrase enzyme of HIV. Integrase enzyme catalyzes the integration of HIV-1 viral DNA into the host cell genome. Once successfully integrated, the proviral DNA then serves as the template for viral RNA synthesis and is maintained as a part of the host cell genome for the lifetime of the infected cell. Several targets for integrase inhibitors have been realized; the integrase enzyme before substrate binding, the viral DNA substrate, and the preintegration complex. During integration, two distinct processes are carried out by this enzyme; 3' processing involving endonuclease activity and the other which is Strand-transfer reaction which the currently approved integrase inhibitors target (Nair et al, 2007). After 3' processing, the integrase enzyme remains bound to the viral DNA as a part of a high-molecular-weight preintegration complex (PIC), which is transported through the nuclear pore complex into the nucleus where integrase catalyzes the strand transfer reaction resulting into the insertion of the 3' processed viral DNA into the chromosomal DNA. It's hypothesized that strand transfer integrase inhibitors bind to the structural intermediate, viral DNA-integrase-divalent metal complex (the preintegration complex) formed after 3' processing, resulting into the stabilized form of the complex that is unable

to bind the chromosomal DNA of the host cell. As a result, the formation of the strand transfer complex that is necessary for viral integration is prevented. Raltegravir is one of the currently FDA approved integrase inhibitors (fig 6).

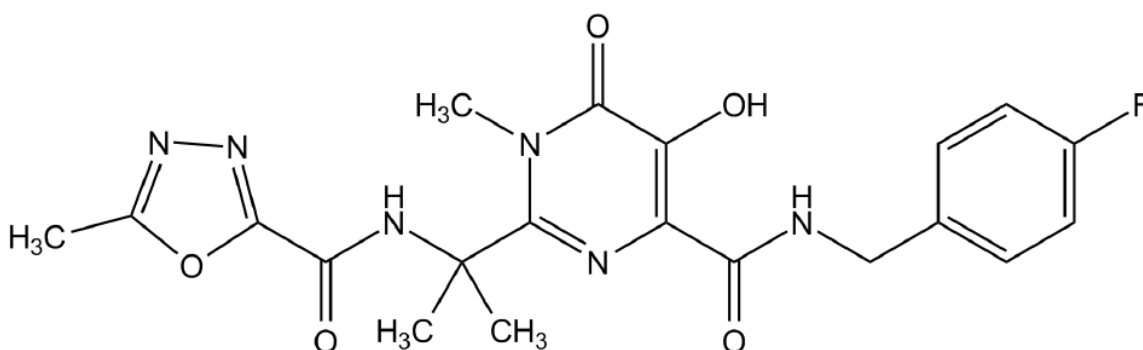


Fig 6: Raltegravir, one of the integrase Inhibitors. Prevents HIV proviral intergration into the host DNA by binding onto the viral DNA-integrase–divalent metal complex. With the inhibitor bound onto the complex, the complex is then unable to bind onto the host chromosomal DNA. The incorporation of the viral DNA into the host genome is then prevented.

2.8 HIV-1 drug resistance mutations

One of the properties of HIV that enables it to develop resistance to ARVs is its extensive genotypic diversity. Diversity is as a result of the error prone nature of reverse transcriptase and the rapid rate of HIV-1 multiplication, with an estimated in-vivo forward mutation rate of 3.4×10^5 per base pair per cycle, resulting in the rapid establishment of extensive genotypic variation (Mansky and Temin, 1995). Failure of exonucleolytic proofreading mechanism of HIV RT enzyme results into substitution of true base pair by a false one in the DNA sequence thereby giving rise to mutant virus. Some of the mutations may lead to the destruction of HIV while others may not cause any significant changes to the gene product, and in this case there will be no effect on the virus. Some mutations might occur and affect genes that code for different important proteins such as the enzymes, structural and regulatory proteins. This may result into altered viral behaviour such as virulence, replication capacity and competence,

cytotoxicity and response to antiretroviral therapy (Kunzi and Groopman, 1993). Resistance mutations can be classified as either major or minor. Major mutations are defined in general either as those that are selected first in the presence of the drug; or those shown at the biochemical or virologic level to lead to an alteration in drug binding or an inhibition of viral activity or viral replication. Major mutations have an effect on drug susceptibility phenotype. In general, these mutations tend to be the primary contact residues for drug binding. Minor mutations generally emerge later than major mutations and by themselves do not have a significant effect on phenotype. In some cases, their effect may be to improve replicative fitness of the virus containing major mutations. However, some minor mutations are present as common polymorphic changes in HIV-1 nonsubtype B (Johnson et al, 2008).

Mutations that confer resistance to antiretroviral drugs have been identified (fig 7 and 8). The International AIDS Society-USA (IAS-USA) reviews and lists mutations that are of clinical importance. The mutations listed below in figure 2 by the IAS (Johnson et al,2008) have been identified by one or more of the following criteria: (1) in vitro passage experiments or validation of contribution to resistance by using site-directed mutagenesis; (2) susceptibility testing of laboratory or clinical isolates; (3) genetic sequencing of viruses from patients in whom the drug is failing; (4) correlation studies between genotype at baseline and virologic response in patients exposed to the drug (IAS-USA,2008).

MUTATIONS IN THE REVERSE TRANSCRIPTASE GENE ASSOCIATED WITH RESISTANCE TO REVERSE TRANSCRIPTASE INHIBITORS

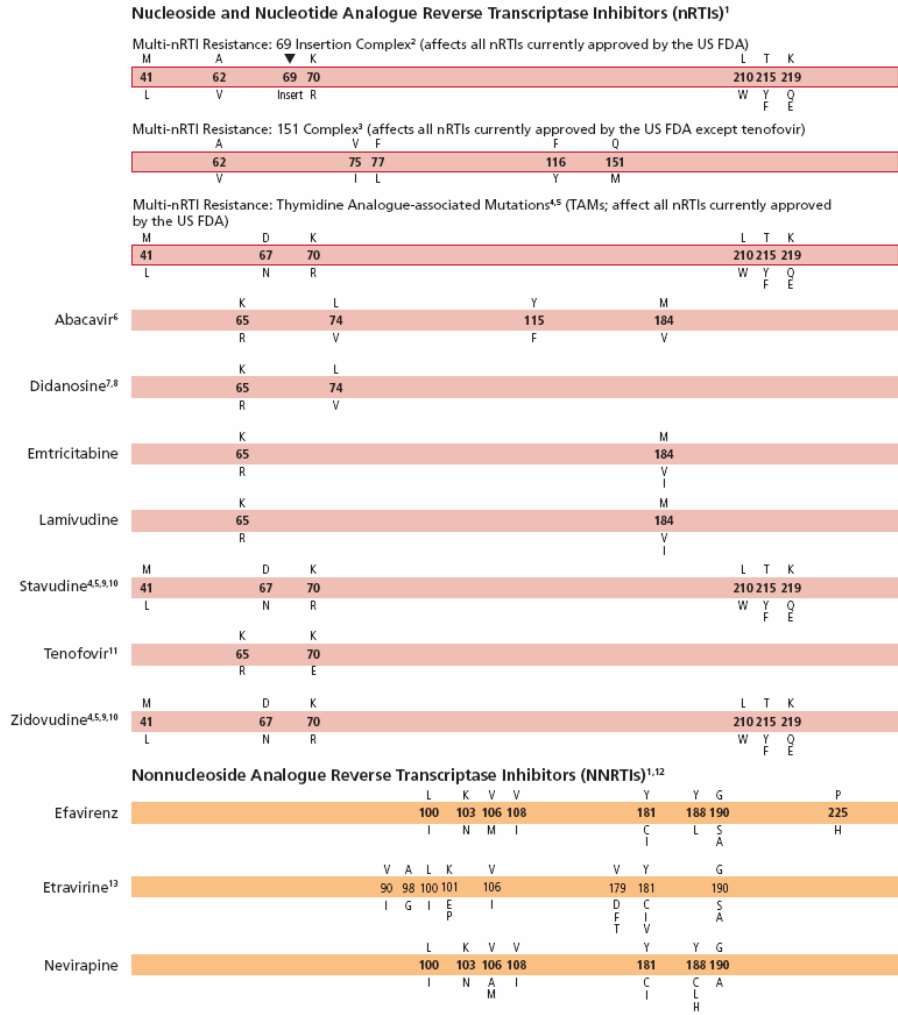
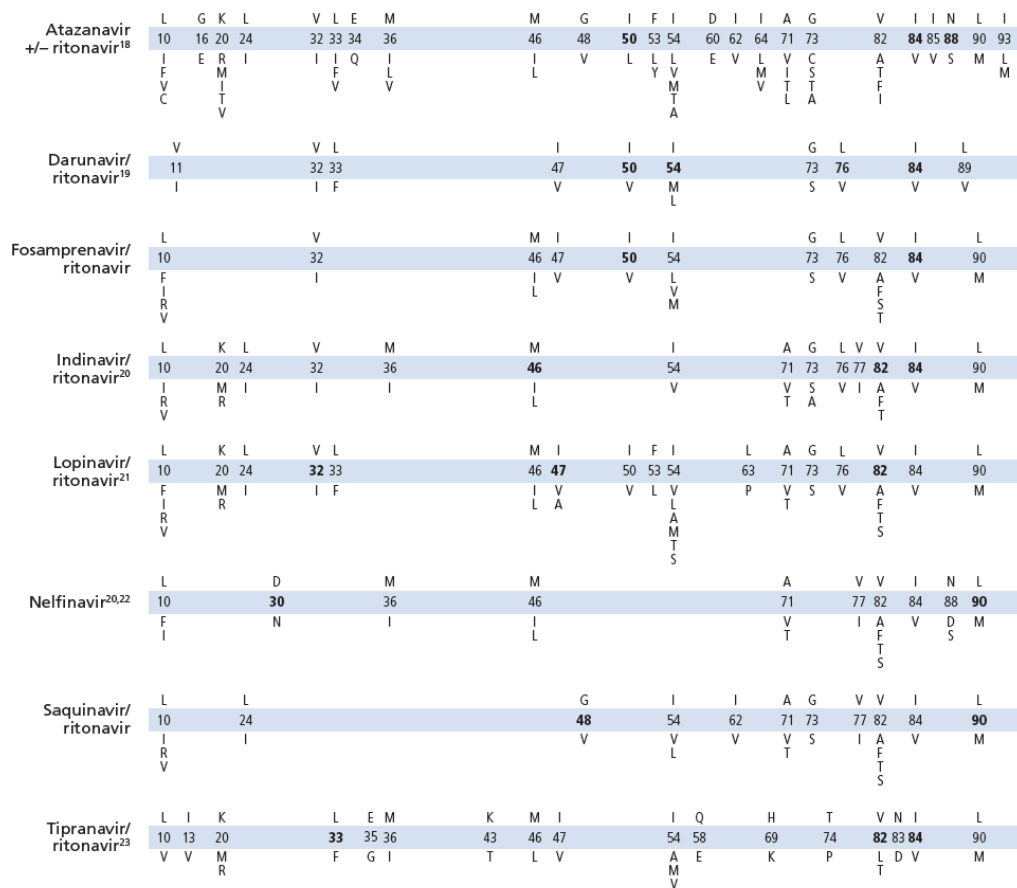
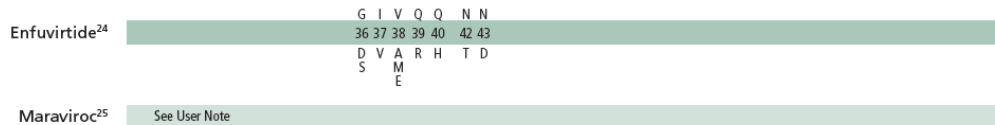


Fig 7: Mutations conferring resistance to Reverse Transcriptase inhibitors: **Reprinted with permission from the International AIDS Society–USA. Johnson VA, Brun-Vézinet F, Clotet B, et al. Update of the drug resistance mutations in HIV-1: Spring 2008. Topics in HIV Medicine. 2008;16(1):62-68. ©2008, IAS-USA. Updated information. Thorough explanatory notes available at www.iasusa.org.** Only major drug resistance mutations have been listed. Most of the mutations occur from aminoacid 41 to 225. mutations conferring resistance to NRTIs are from codon 41 to 225 while that of NNRTI are from codon 90 to 225. Etravirine, a new NNRTI has a high efficacy against the mutants resistant to all other drugs in the NNRTI class. Thymidine analogue Mutations (TAMs) are a subset of Nucleoside Analogue Mutations (NAMs) that are selected by the thymidine analogues zidovudine and stavudine and are associated with cross-resistance to all NRTIs currently approved by the US FDA.

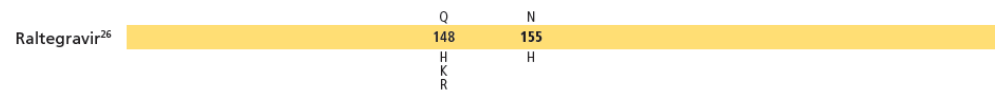
MUTATIONS IN THE PROTEASE GENE ASSOCIATED WITH RESISTANCE TO PROTEASE INHIBITORS^{14,15,16,17}



MUTATIONS IN THE ENVELOPE GENE ASSOCIATED WITH RESISTANCE TO ENTRY INHIBITORS



MUTATIONS IN THE INTEGRASE GENE ASSOCIATED WITH RESISTANCE TO INTEGRASE INHIBITORS



Amino acid abbreviations: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

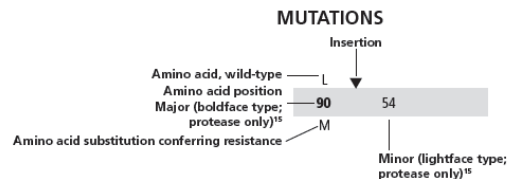


Fig 8: Mutations conferring resistance to PIs, Entry and Integrase inhibitors: Reprinted with permission from the International AIDS Society–USA. Johnson VA, Brun-Vézinet F, Clotet B, et al. Update of the drug resistance mutations in HIV-1: Spring 2008. Topics in HIV Medicine. 2008;16(1):62-68. ©2008, IAS–USA. Updated information. Thorough explanatory notes available at www.iasusa.org. Major and minor drug resistance mutations have been listed. Ritonavir is used to boost other PIs and not a PI on its own. Resistance to Enfuvirtide occurs in the envelop gene from amino acid 36 to 43. Coreceptor usage and density may affect susceptibility to enfuvirtide. Maraviroc activity is limited to patients with only CCR5 co-receptor tropic viruses. Mutations in the HIV-1 gp120 can also lead to reduced susceptibility to Maraviroc.

2.9 Mechanism of HIV drug resistance

The emergency of resistant virus is one of the main reasons for failure of antiretroviral therapy (Hoffman and Kamps, 2003). This often limits the drug options available for treatment especially where there is cross-resistance.

Because of the poor replication fidelity of the reverse transcriptase enzyme of HIV, there is a high error rate estimated at about 10^{-4} to 10^{-5} mutations per base pair per replication cycle (Mansky and Temin, 1995) This results into high volume of viral diversity or species known as quasi species and these are found circulating within a single host (Peeters, 2001). These species are slightly different in their genetic make up. From these quasi species can be found the different virus that is resistant to the different drugs especially when introduced in suboptimal doses. The wildtype virus is assumed to have the highest replication capacity in the absence of drugs while the drug resistant mutant virus will be fitter in presence of the drug (Mammano et al, 2000). When the selective drug pressure is removed the wildtype usually reemerges (Briones et al, 2001).

For the NRTI, the resistance mutations in the reverse transcriptase enzyme result into structural changes leading to loss of affinity of the inhibitors for the active site. Binding of physiological nucleotides is favoured over that of the drug (de Mondeza *et al*, 2002). Another mechanism involves the removal of the chain terminator by phosphorylysis via Adenosine triphosphate or pyrophosphate. These are the nucleoside-associated mutations (de mondeza & Hoffman and Kamps, 2003). Some inserts at the p6 region within the gag gene may favor virus escape from NRTI through a greater accumulation of reverse transcriptase molecules per virion (de Mondeza *et al*, 2002.). Phosphorylysis leads to cross resistance between NRTIs, the degree of which may be AZT, D4T>ABC>ddC, ddi>3TC (Hoffman and Kamps, 2003).

For the NNRTI, resistance mutations only result into structural changes leading to loss of affinity of the inhibitors for the enzyme. Resistance often results from single mutations near the drug pocket binding, K103N being the most frequent mutation (Soriano *et al.*, 2002).

Resistance mutations to protease inhibitors usually develop slowly as several mutations must accumulate (Hoffman and Kamps, 2003). Some of these mutations result into structural changes in the substrate cleft that lead to a reduction in the drug binding affinity (Soriano, *et al* 2002). Also mutations at several of the protease cleavage sites have been associated with resistance.

Entry inhibitors include many compounds that are able to block HIV entry into the cells at different steps such as compounds that inhibit gp120–CD4 binding (Leonard *et al*,1990), gp120–co-receptor binding (Wu *et al*,1997 and Picard *et al*,1997), and those that bind to the HR1 region of gp41 of HIV (Weiss, 2003). Currently two entry inhibitors have been approved by the FDA: Maraviroc, a CCR5 antagonist which has been recently approved (www.avert.org). Enfuvirtide, is a fusion inhibitor which binds to HR1 to prevent the fusion process between the viral and host cell membrane (Weiss, 2003).

Two main resistance pathways are theoretically possible for resistance to CCR5 and CXCR4 antagonists to emerge. The first is a shift in co-receptor usage and the second results from changes in the HIV envelope genomic regions which allow the interaction between gp120 and the co-receptor despite the presence of the inhibitor (Briz *et al*, 2006). Most CCR5 antagonist-resistant strains continue to use CCR5 co-receptor rather than shifting to CXCR4. Multiple mutations within different regions of HIV gp120 (V3, C2, V2, C4) account for the drug-resistant phenotype (Trkola *et al*,2002). The most relevant aminoacid changes were likely to be in the V3 loop of the gp120 glycoprotein (Shawn *et al*,2003). For CXCR4 antagonists, mutations in the HIV gp120 V3 domain seem to account for the loss of susceptibility to many of these compounds.

Much as a mechanism for resistance to antagonists to CCR5/CXCR4 which involves a switch in correceptor has not been shown as the major one, it might still be possible.

Resistance to Enfuvirtide involves changes in amino acids from position 36 to 45 within the HR1 region of gp41 (Wei *et al*, 2002 and Poveda *et al*, 2004).

Most HIV infected patients in the developing world are mainly treated with reverse transcriptase inhibitors. Nevirapine, a non-nucleoside reverse transcriptase inhibitor plus two other Nucleoside reverse transcriptase inhibitors is the first line of treatment regimen

in developing countries (Kantor *et al.*, 2002). This could be the reason for the high prevalence of drug resistance mutations in the reverse transcriptase region of HIV1 treated Ugandans as shown by Richard *et al* (2004).

2.10 HIV subtypes and their role in drug resistance

Most HIV infections are treated with antiretroviral drugs, and the highly active antiretroviral therapy being the most recommended combination. This mainly involves using a protease inhibitor, nucleoside reverse transcriptase inhibitor as well as a non-nucleoside reverse transcriptase inhibitor. Much as these ARVs have been able to suppress HIV replications, one of the major challenges has been development of HIV drug resistance. World over, HIV, through its high replication capacity and low proof reading mechanism of its gene, has been able to diversify into different subtypes geographically. Further more most of the drugs have been designed to target the subtype B virus. Very little data are available as to how subtype diversity may affect drug susceptibility and resistance.

Different subtypes have shown different response to drugs. Palmer *et al*,1998, studied the susceptibility profile of HIV-1 subtypes A, B, C, D, and E isolates from treated and untreated patients to zidovudine (ZDV), lamivudine (3TC), didanosine (ddI), nevirapine (NVP), foscarnet (PFA), and ritonavir (RNV). All the subtypes had comparable susceptibility except D which showed reduced susceptibility to the drugs than the others.

Some in vivo studies in Brazil reveal increase in the drug resistance within the drug naïve patients, and with different prevalence of resistance among the B and non B HIV subtypes in Brazil (Ivone *et al*, 2004). Some of the mutations were more prevalent in the non B subtypes than in the B. Spira *et al*, 2003 compared the drug resistance polymorphisms of subtypes B and C from treatment naïve patients and showed a high level of secondary resistance polymorphisms with the subtype C virus isolates. These polymorphisms occurred within the reverse transcriptase gene at sites linked to resistance to NNRTIs and NRTIs. These polymorphisms which could only confer resistance in presence of another drug resistance mutation included R211K and L214F (conferring

resistance to NRTI), A98S and E138K (conferring resistance to NNRTI) and M36I (conferring resistance to PI) (Loomba *et al*,2002). Further invitro studies have shown that NNRTI drug resistance mutations emerge within subtype C virus at a much lower drug concentration than with subtype B, (Loomba *et al*,2002) and this could be one of the contributing factors to the high level of drug resistance in developing countries which use sub-optimal therapy because of the prohibitive drug costs. Additionally, these invitro studies revealed that subtype C isolates were more likely to develop resistance rapidly than the B. For instance it was shown that C developed resistance to NVP or DLV 8 or 9 weeks and 13 weeks with efavirenz while subtype B isolates took at least 15 weeks with NVP or DLV and 30 weeks with EFZ (Loomba *et al*, 2002).

In a cohort of women who had received single dose nevirapine in the PMTCT program, resistance to nevirapine was higher in the HIV-1 subtype C than with either A or D subtypes (Elshleman *et al*, 2005). Nevirapine resistance in subtype D was higher than that in A. These subtype C variants were also more likely to develop cross resistance to all the NNRTIs making it impossible to use any of these drugs in any future PMTCT with these patients.

Some new drug resistance mutations previously unknown have been characterized in non B subtypes. S98I and A98S mutations in subtype C are associated with resistance to nevirapine (Loomba *et al*, 2002). V106M mutation which was exclusively selected for by efavirenz in tissue culture isolates was also found in some patients on treatment with efavirenz (Brenner *et al.*, 2003). This mutation conferred high-level cross-resistance to all Non-nucleoside reverse transcriptase inhibitors in tissue culture isolates. V106A is listed as a nevirapine-specific mutation (Brenner *et al.*, 2003). Gao *et al.*, 2004, have characterized a mutation that displayed a high-level resistance to nevirapine and delavirdine in the D subtype of HIV-1 from a Ugandan drug naive infant. This isolate did not have any of the amino acid residues in the reverse transcriptase region of HIV-1 typically associated with Non-nucleoside reverse transcriptase inhibitors. HIV-2 and Group O variants of HIV-1 have been shown to be resistant to all the NNRTIs. This has been mainly due to the presence of Y181I mutation among the group O and the Y181C

mutation in HIV-2 (Quinones *et al*, 1998, and Descamps *et al*, 1997). Treatment of these infections is thus limited because of the limited available ARV options.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study design

This was a cross sectional study of 104 patients who reported to be naïve to antiretroviral therapy. The proviral deoxyribonucleic acid (DNA) of HIV-1 was extracted from the PBMC of the patient samples and its POL gene amplified using the polymerase chain reaction (PCR). The samples were then sequenced for drug resistance mutations. 70 patient samples were successfully sequenced and analyzed for resistance to reverse transcriptase inhibitor drugs while 34 patient samples were analyzed for resistance to protease inhibitors.

3.2 Study Population

This study was part of a larger laboratory based project entitled “*The HIV-1 antiviral Outcomes and Resistance in Africans in Uganda, Zimbabwe and Cameroon.*” The Principal investigators of the study were Dr Peter Mugenyi from Joint Clinical Research Centre, Kampala and, Dr Robert Salata and Dr Eric Arts, Case Western Reserve University, United States of America. My study addressed part of the specific aims of the larger lab based study which compared treatment outcomes among patients on clinical studies and those on treatment from national programs. The samples were obtained with permission from another study titled “The effect of Hormonal Contraception on HIV Genital Shedding among Women with Primary HIV Infection (Genital Shedding Study or GS study)”. The GS study examined the impact of hormonal contraception on HIV genital shedding in both acute and chronic HIV infections. The GS study was an ancillary study of the Hormonal Contraception and the Risk of HIV Acquisition (HC-HIV) study. The GS study was conducted among women who became HIV infected while participating in the HC-HIV study. See appendix for permission to use samples.

Patient enrollment: As part of the GS study, any HIV positive participant in the HC-HIV study cohort was immediately reported to the GS study coordinator. The HC-HIV study participant was immediately traced, and requested to return to the clinic to receive her HIV results, receive post test counseling, and have blood drawn for an HIV retest. When

the participant's re-test results indicated she was HIV positive, she was then offered enrollment into the GS study. HIV retesting was done within 15 days from the time of the 1st seroconversion. Follow up schedules for the participants are every three months, starting with the seroconversion visit.

The primary objective of the HC-HIV study was to measure the effect of combined oral contraceptives and Depot Medroxyprogesterone Acetate (DMPA) use on the acquisition of HIV infection by comparing the rate of infection among women using COCs and DMPA with the rate among women not using hormonal contraception. Participants were recruited from three family planning clinics situated in Kampala, Uganda. Two clinics were in Mulago hospital and the other was the international planned parenthood federation clinic. All combined were supposed to enroll 2,270 HIV negative women over a 15 months period.

3.3 Enrollment criteria

3.3.1 Inclusion criteria. Patients included in our study were,

HIV seropositive

Naïve to ARVs at the time of joining the study

Known period of seroconversion

Previously closely monitored for HIV infection

CD4 count >250,

Age; 18-45years

3.3.2 Exclusion criteria; Patients were excluded from the study if ,

Indeterminate serostatus,

Pregnant or intended to become pregnant during the subsequent 12 months,

Patients who had had blood transfusion 3 months prior to enrollment.

3.4 Study site

The laboratory work was done at the Joint Clinical Research Centre Kampala.

3.5 Study period

The study took approximately 5 months from May 2008 to September 2008.

3.6 Sample size and Sampling

For this study, a total of 104 PBMC samples stored from all HIV-1 seroconverter patients in the GS study were used. In determining resistance to NNRTIs and NRTIs, samples from 70 patients that were successfully sequenced for the RT region of HIV-1 were used. We believe the sample size is fairly adequate as most previous studies have used about 60 to 100 patients to determine resistance prevalences (Pillay et al, 2007, Chonlaphat et al, 2008 and Baker et al, 2007). For resistance to PIs, 34 samples were successfully amplified and used. Some of the samples weren't successfully amplified or sequenced possibly because of low viral load where by inadequate viral DNA concentration was available for amplification and possibly poor primer incompatibility for sequencing. Each seroconverter was enrolled in to the GS study and all enrollments from 2001 to 2007 were used for this study.

3.7 Handling of samples.

The GS study collected blood (EDTA) from the patients and then extracted peripheral blood mononuclear cells (PBMC). These were stored at -70C or in liquid nitrogen. PBMCs were used in the subsequent steps in this study.

3.8 Ethical considerations and consent to the studies.

These were catered for by the studies whose samples were used. The GS study had a full IRB approval from AIDS Review Board. Women in the GS study underwent a consent process informing them of the procedures to be followed. The Senior social scientist of the GS study made sure that the study was conducted in accordance with the local IRB, mentioned above and in a manner that was sensitive to the culture in Uganda. Participants consented to the HC-HIV and GS studies and their samples to be used for any other future studies that may arise.

3.10 Laboratory Procedure

3.10.1 DNA extractions from PBMCs.

DNA was extracted from PBMC according to the instructions of the Qiampr DNA Blood mini kit from QIAGEN Sciences, Maryland 20874, USA.

3.10.2 Genotypic drug resistance

This was done following the different steps below using the DNA extracted from the PBMC. Reagents were obtained from Invitrogen Life Technologies, California, USA.

3.10.2.1 PCR amplification of both the protease and the reverse transcriptase gene.

Two PCRs were performed i.e. external and nested.

a. External PCR: 50 µL PCR reaction

The master mix was prepared as follows.

- i. 5µL of 10X PCR buffer (with 1.5 mM final MgCl₂),
- ii. 1.0µL 10 mM dNTPs,
- iii. 1.0µL primer RTA9 (25 pmol/µL), and 1.0 µL of primer PS5 (25 pmol/µL),
- iv. 0.25 µL Taq DNA polymerase (5 U/µL), and
- v. 39 µL water.
- vi. 47.5 µL of master mix were added to each tube followed by 2.5 µL of proviral DNA (This was the HIV-1 DNA extracted from the PBMC).
- vii. The following conditions were used to amplify the DNA using the polymerase chain reaction (PCR): 94°C for 4 min, [94°C 30 s, 55°C 30 s, 72°C 1 min] for 35 cycles, 72°C for 5min as final extension and 4°C hold.

b. Nested PCR: 50 µL PCR reaction.

- i. 5µL of 10X PCR buffer (with 1.5 mM final MgCl₂),
- ii. 1µL 10 mM dNTPs,
- iii. 1µL primer RTA8 (25 pmol/µL), and 1.0 µL of primer PS3 (25 pmol/µL),
- iv. 0.25 µL Taq DNA polymerase (5 U/µL), and
- v. 39 µL water.

- vi. 47.5 μL (see above) of master mix were added to each tube followed by 2.5 μL of external PCR product
- vii. 5 μL of each nested PCR product (plus loading dye) were run on a 1% non-denaturing agarose gel alongside a molecular weight marker.
- viii. The remaining 45 μL of nested PCR product were purified using QIAquick PCR purification kit (QIAGEN Sciences, Maryland 20874, USA) once the correct size of the PCR product was identified .

3.10.2.2 DNA sequencing

This was done using the CEQ 8000 sequencer from Beckman Coulter.

The sequence data generated from the sequencer was analysed using the BioEdit programme (Version 7.0.5). The Stanford drug resistance database (www.hivdb.stanford.edu), was used to identify and interpret the drug resistance mutations.

3.11 Data collection and statistical analysis

The prevalence of drug resistance was determined by getting the number of patients with at least one drug resistance mutation and dividing that by the total number of patients in the study.

To determine HIV drug resistance in each of the subtypes, the number of patients with at least one drug resistance mutation in each of the subtype was divided by the total number patients in that subtype.

For specific aim IV, to determine whether there is an association between subtype and emergence of resistance, subtypes were determined and the proportions resistant to the drugs compared. Using SPSS, the chi square method was used to determine whether there was any association between subtype and HIV drug resistance.

The phylogenetic analyses of the patient sequences were performed and HIV subtypes determined using Clustal X alignment program Version 1.83.

CHAPTER FOUR: RESULTS

4.1 Demographics of study population

These were females in the age group of 18 to 45 years old participating in one study that was looking at impact of hormonal contraception on HIV acquisition in both acute and chronic infections. These clients came from both urban and semi urban environment.

4.2 HIV drug resistance to Protease Inhibitors

Of the 34 patients whose protease gene of HIV DNA was successfully sequenced, each patient had at least one mutation or polymorphism associated with reduced response to therapy. All these mutations were minor PI drug resistance mutations (www.hivdb.stanford.edu). In the general cohort the highest prevalence of resistance mutations or polymorphisms was 67.6%, 61.8% and 47.0% for mutations M36I, I13V, H69K respectively as seen in (Table 1). Mutations I62V and D60E occurred at the lowest level with a prevalence of 2.9% each.

Table 1
Prevalence of resistance polymorphisms previously shown to be associated with reduced response to treatment with Protease inhibitors.

Drug resistance Mutation	Number of Patients (per subtype)			Percentage of Total patients
	A	C	D	
M36I	12	3	8	67.6
I13V	10	0	11	61.8
H69K	14	2	0	47.0
G16E	5	0	1	17.6
K20R	2	0	1	8.8
L10V	4	0	0	11.8
I62V	0	0	1	2.9
D60E	0	0	1	2.9

Total number of patients whose HIV DNA was successfully PCR amplified and sequenced for Protease drug resistance was 34.

4.3 HIV-Subtypes in the protease region

HIV subtypes were determined using Clustal X alignment program Version 1.83 (fig 11). For resistance to protease inhibitors, the protease gene was used to determine subtypes. It was found that 15 of the individuals had subtype A virus, 3 harboured subtype C and 16 had subtype D virus giving a prevalence of 44.1%, 8.8% and 47.0%. The prevalence of HIV drug resistance per subtype for each of the mutations or polymorphisms was significantly highest in subtype A followed by D (fig 9). H69K, which was the highest polymorphism detected was unique to subtype A and C. The polymorphism with the highest frequency in subtype A was H69K with a prevalence of 93.3% while I13V was the commonest polymorphism in subtype D with frequency of 68.8% in that subtype. 8 individuals carried only one mutation and all these were of subtype D, giving a total prevalence of 23.5%. 8 individuals of the subtype D that carried at least 2 drug resistance mutations give a proportion of 50% in that subtype while all individuals in subtype A carried at least 2 drug resistant mutations, giving a proportion of 100%.

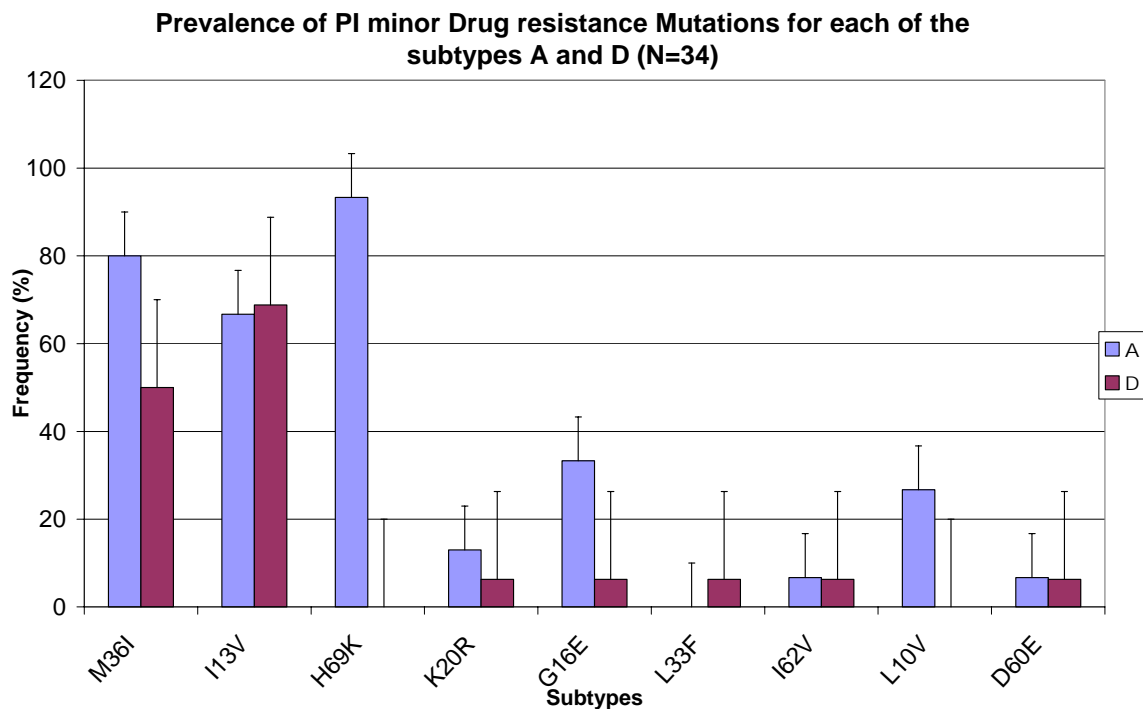


Fig 9. There is a high number of mutation or polymorphism in subtype A than in D.

4.4 HIV drug resistance to Reverse transcriptase inhibitors

The total number of patients sequenced for reverse transcriptase gene was 70. Again subtyping was determined, based on the reverse transcriptase gene using the Clustal X alignment program (fig 12) giving subtypes A (45), C (3) and D (22). For the NRTI, five drug resistance mutations previously shown to be associated with reduced response to therapy were found in these patients. Of these, M184V was the only major drug resistance mutation while the rest (V118I, E44D, D67N and T215S) were minor drug resistance mutations (Table 2). All the mutations or polymorphism associated with reduced response to NRTI therapy were from subtype A virus, with the exception of E44D which was from patients harbouring subtype D virus. Two patients carried a major drug resistance mutation for NRTI resistance which was M184V. V118I had the highest prevalence. Two patients harboured mutations associated with transmitted drug resistance: M184V was harboured by the two patients and T215S harboured by one of the patients. All these patients had subtype A virus. Prevalence of transmitted resistance was 2.9%.

Four mutations conferring resistance to NNRTI were found, of which K101E, K238T and K103N were major drug resistance mutations while P225H was a minor drug resistance mutation (table 2). All the patients with the major drug resistance mutations, K101E and K103N, belonged to subtype D while only one patient though harbouring two NNRTI mutations, K238T and P225H, belonged to subtype A. This patient is one of the two harbouring major mutation M184V, for transmitted drug resistance (Table 2).

Table 2
Prevalence of individual drug resistance mutation associated with reduced response to reverse transcriptase inhibitors

Mutation	Number of Patients for each subtype			Prevalence (%) as Percentage of total patients
	A	D	C	
NRTI-Mutations				
V118I	3	0	0	4.3
E44D	0	1	0	1.4
D67N	1	0	0	1.4
M184V^a	2	0	0	2.9
T215S ^a	1	0	0	1.4
NNRTI-Mutations				
K101E	0	2	0	2.9
K238T	1	0	0	1.4
P225H	1	0	0	1.4
K103N	0	1	0	1.4

^a Marker mutation for transmission of drug resistance

Total number of patients sequenced for reverse transcriptase gene was 70. Note: One patient had five mutations, M184V, K103N, P225H, K238T and T215S. In BOLD are the major drug resistance mutations.

Three drug classes were considered: NRTI, NNRTI and the reverse transcriptase inhibitors (RTI) which represented both the NRTI and NNRTI as a single group of drugs targeting the reverse transcriptase gene of HIV-1. Prevalence of drug resistance for all the three drug classes was calculated (table 4). Resistance to NRTIs was highest among subtype A, with 6 individuals harbouring resistance mutations while resistance to NNRTIs was highest in subtype D. Resistance to RTI was highest in subtype A than in D. The frequency of resistance for each of these drug classes (NRTI, NNRTI and RTIs) among this cohort was 10.0, 4.3 and 12.9 respectively (Table 3).

4.5 HIV subtypes and resistance to the drug classes (NRTIs, NNRTIs, RTIs)

The prevalence of drug resistance for each of the subtypes A and D was calculated so as to find out whether there is an association between these two subtypes and drug resistance (fig 10). Subtype A had a higher prevalence of resistance to NRTIs (13.3%) as compared to D (4.5%) while resistance to NNRTIs was higher in subtype D (9%) than in A (2.2%). Generally Subtype A was more resistant to NRTIs while D was more resistant to NNRTIs though none of this was statistically significant. From fig 10, resistance to RTIs was almost the same for both subtype A and D.

Table 3
Prevalence of drug resistance to Reverse transcriptase inhibitor drug classes

Drug class	Number of patients for each of the subtypes			Total number Patients with resistant mutation	Prevalence as a percentage of all patients in the study
	A	D	C		
NRTI	6	1	0	7	10.0
NNRTI	1	2	0	3	4.3
RTI	6	3	0	9	12.9

Note: RTI includes both NNRTIs and NRTIs all grouped together as one class of drugs. The total number of patients was 45, 22 and 3 for subtypes A, D and C respectively. One patient fell into two groups i.e. One patient from subtype A had both an NNRTI and NRTI drug resistance mutation, hence the total number of patients with resistance to RTI was 6 instead of 7.

**Frequency of HIV drug resistance Mutations for each of the Subtypes A and D
(N=67)**

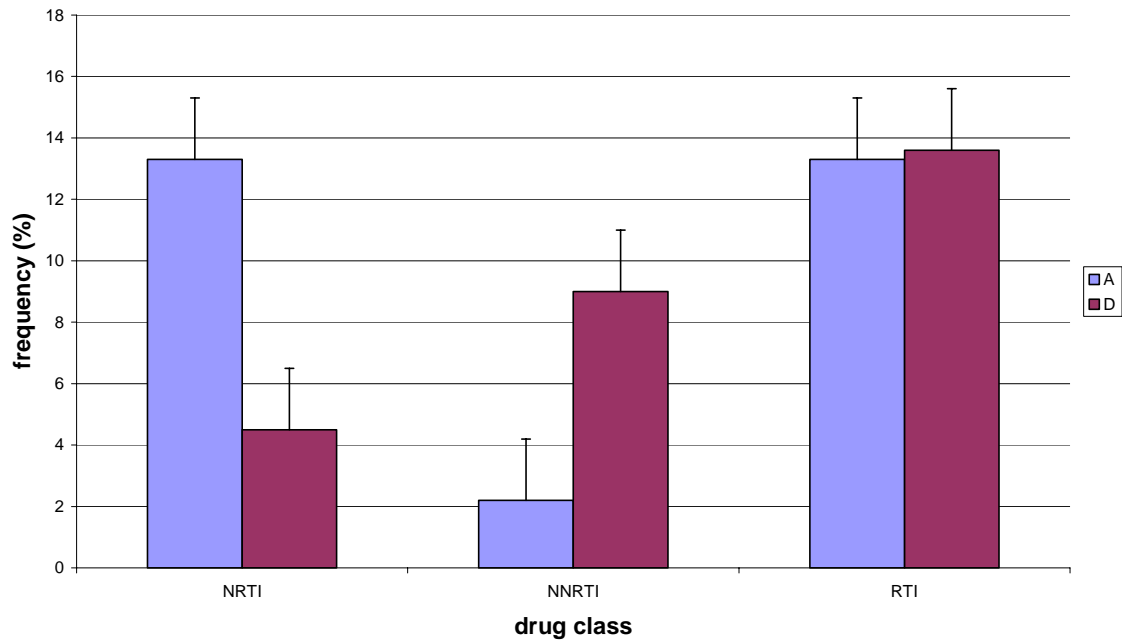


Fig 10. shows the prevalence of drug resistance to RTIs, NNRTI, and NRTIs for each of the subtypes A and D. Total number of patients for both subtypes A and D was 67. The total number patients for subtypes A and D was 45 and 22 respectively Subtype A had the highest level of drug resistance to NRTIs . Generally Subtype A was more resistant to NRTIs while D was more resistant to NNRTIs though none of this was significant.

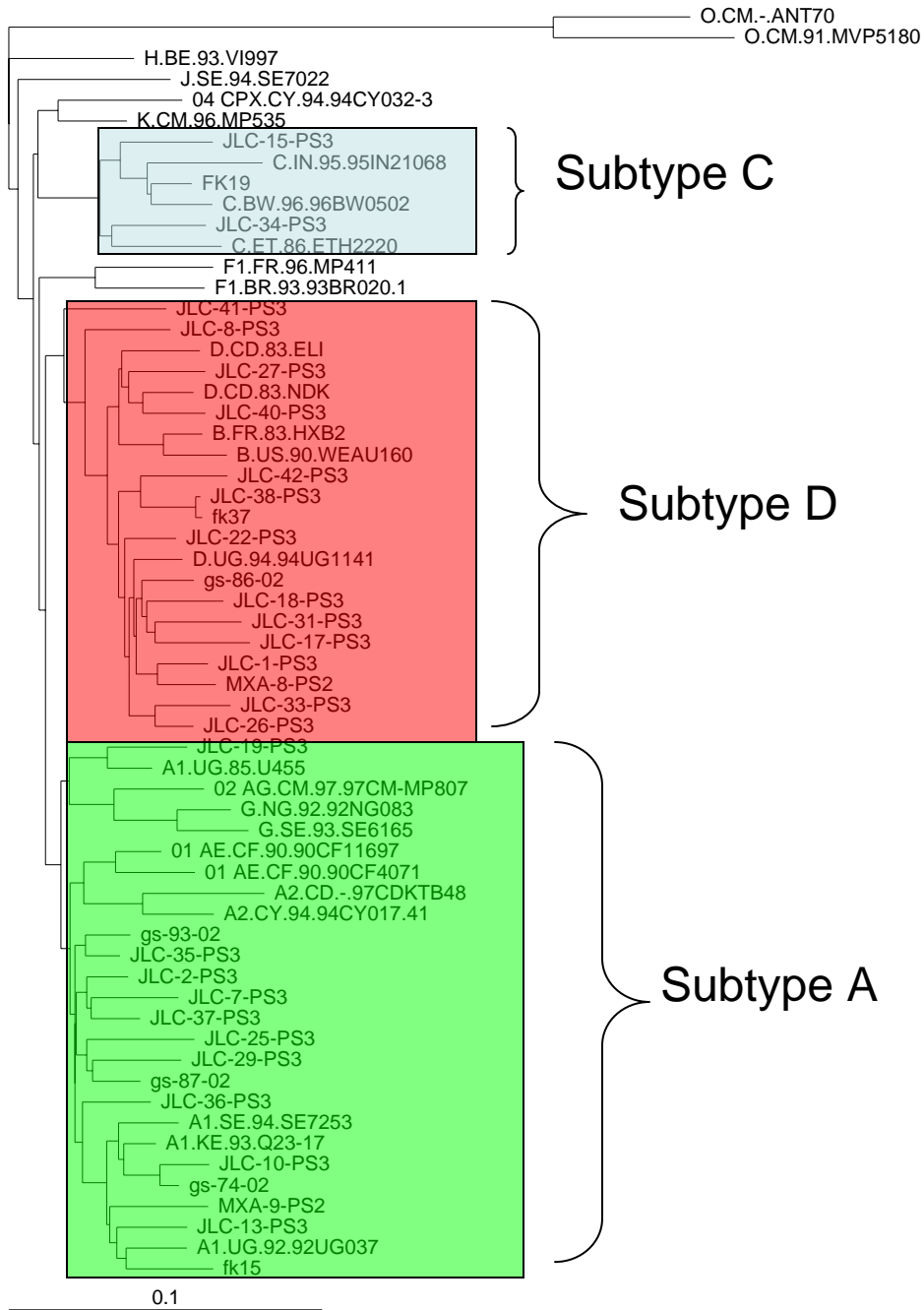


Fig 11 Phylogenetic tree showing HIV subtypes in the Protease region. There were 15 patient viral sequences clustering with known strains from subtype A, 3 clustering with known viral strain from subtype C and 16 clustering with subtype D. All samples are indicated with JLC, gs or Fk identification numbers. Other identifications letters or numbers show the different subtypes and how they are closely related to the samples.

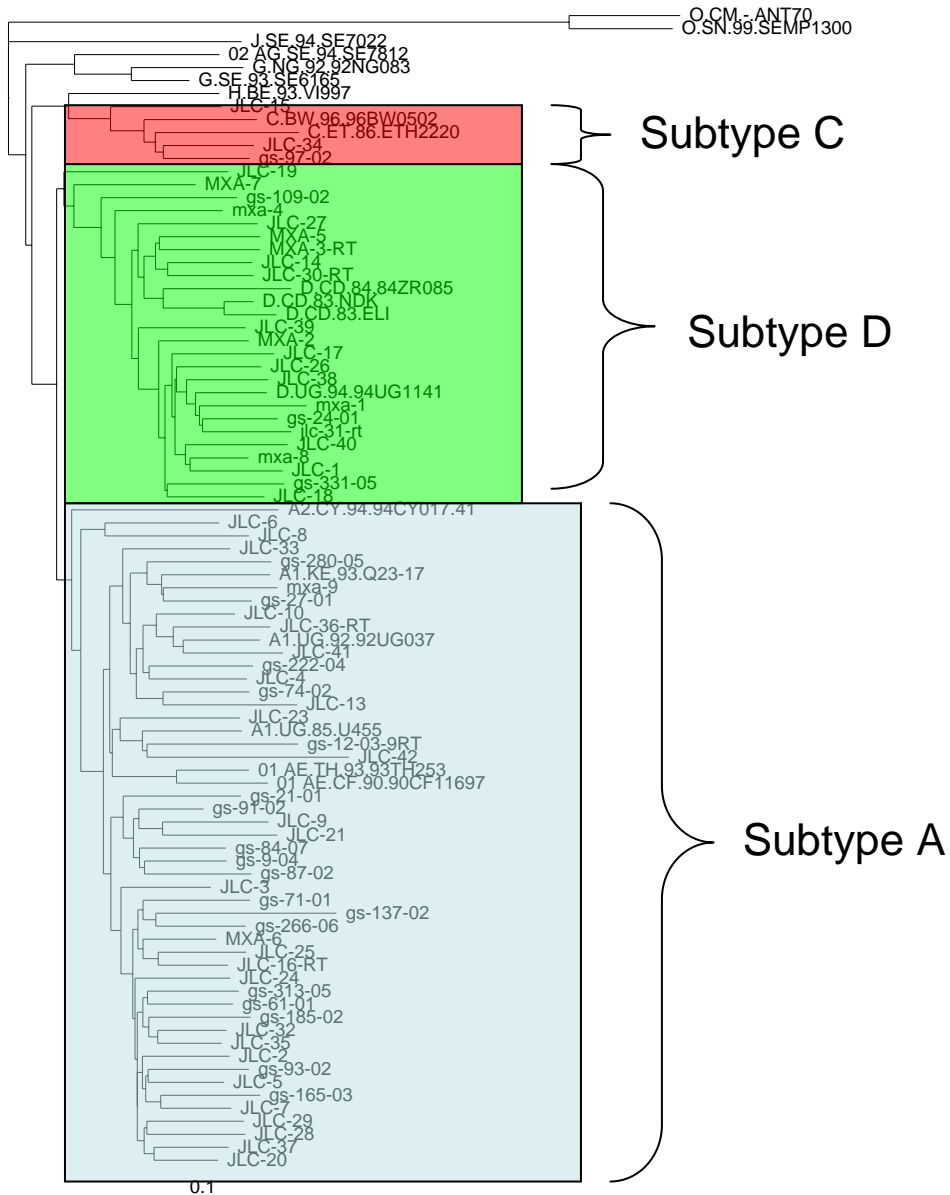


Fig 12 Phylogenetic tree showing HIV subtypes in the Reverse transcriptase region of HIV. 45 sample patients clustered with the known subtype A strains, 22 patient viral sequences clustered with the known subtype D sequences, and 3 patients viral sequences clustered with subtypes C sequences. All samples are indicated with JLC, gs or Fk identification numbers. Other identifications letters or numbers show the different subtypes and how they are closely related to the samples.

CHAPTER FIVE: Discussion, Conclusion and Limitation to the study

5.1 Discussion and conclusion

Mutations conferring resistance can be classified as major (primary) or minor (secondary). Major mutations are defined in general either as those that are selected first in the presence of the drug; or those shown at the biochemical or virologic level to lead to an alteration in drug binding or an inhibition of viral activity or viral replication. Major mutations have an effect on drug susceptibility phenotype. Minor mutations generally emerge later than major mutations and by themselves do not have a significant effect on phenotype. Though no major PI resistance mutation was found, at least each of the 34 samples sequenced in the protease gene had a minor PI resistance polymorphism. The absence of major PI mutations could be due to their limited use in the Uganda where they are reserved for patients mainly failing on the first line therapy. Other studies have also found few or no major drug resistance polymorphisms to PIs in settings where their use is limited (Chonlaphat et al, 2008 and Hart et al, 2003). PI resistance was significantly higher in Subtype A than Subtype D ($P=0.001$, X^2). Both primary and secondary mutations have an impact on the virus. Minor drug resistance polymorphisms contribute to the reduced susceptibility of virus to drugs when there is a major mutation. As resistance to PIs increases during treatment, the replication capacity of the virus decreases. Emergence of primary genotypic mutations within the protease gene have been associated to the compromised replication capacity of the virus while emergence of secondary mutations within the same gene was associated with more-gradual changes in both phenotypic resistance and replication capacity (Barbour et al, 2002). Minor mutations compensate for the reduced fitness of the virus that could have resulted due to the introduction of the primary mutations and further more, these compensatory or minor mutations are similar for all PIs. On the other hand, primary mutations may be unique for the different PIs (Erickson et al, 1999). Therefore when resistance emerges to both subtypes, subtype A which has more minor resistance polymorphism will in the end be more resistant to the PIs because it will have a better replication capacity than its counterpart D. Some data suggest that prior M36I and L10I/V mutations are associated with a more rapid fall in PI sensitivity during treatment.

Data from phase II and III clinical trials showed that accumulation of mutations, L10V, I13V, K20M/R, L33F, E35G, M36I, K43T,I47V, I54A/M/V, Q58E, H69K, T74P, V82L/T, N83D, and I84V, was associated with reduced response to protease inhibitors (Johnson et al, 2008).

Fewer mutations or polymorphisms were identified in the reverse transcriptase gene than protease. However, in some cases there were major drug resistance mutations which included M184V, K103N, and K101E. M184V is a marker mutation for transmitted drug resistance (Vandamme et al, 2004) in naïve patients and this gives a prevalence of 2.9% for the transmitted resistance. The prevalence of NRTIs resistance mutations was higher for subtype A (13.3%) than D (4.5%), though not statistically significant. Conversely prevalence of NNRTI resistance mutation was higher for subtype D (9.0%) than A (4.5%) though not statistically significant. The prevalence of resistance to all the reverse transcriptase inhibitors was 12.9%. Pillay et al, 2007, have given a prevalence of 9% among drug naïve patients. This is in agreement with this study whose prevalence is 12.9%. Prevalence of resistance among the drug naïve patients is expected to rise as more people access ART because then there will even be more transmission of mutant virus , there by increasing prevalence of resistant virus.

In agreement with Pillay et al, 2007, subtype was not significantly associated to resistance to reverse transcriptase inhibitors or their smaller classes (NNRTI and NRTI) in naïve patients. However this contradicts another study which showed that resistance to NRTIs was higher in subtype A and resistance to NNRTIs was higher for subtype D (Baker et al, 2007). Our results, however, at a non significant level do agree with Baker et al, 2007, that resistance to NRTIs was higher in subtype A and resistance to NNRTIs was higher for subtype D. One of the reasons for the disagreement could be because Baker et al, 2007 determined viral subtype basing on the Nef and Gag region and not the reverse transcriptase region, the target for the RTIs as we have done. Recombination has been shown in HIV where by one of the genes may be of a different subtype. For instance in the GAG region it could have Subtype A while in the RT region, it could have D. This can bring about false numbers of subtypes depending on which region is used.

Much as these results have shown that resistance to NNRTIs is not significantly associated to subtype D, though the proportion of resistant individuals are higher for D, it could however be used to predict the likelihood of the emergence of these mutations in individuals on treatment. For instance, women who were given a single dose of NVP for prevention of mother to child transmission of HIV, after 6 to 8 weeks, NVP resistance mutations were detected at a higher rate in women with HIV-1 subtype D than those with subtype A (Eshleman et al, 2004). Therefore for a country like Uganda where we use NVP in PMTCT, caution must be taken when treating these patients using combination having NVP in it.

Several investigators have documented drug resistance strains in patients newly infected with HIV due to transmitted drug resistance (Imrie et al, 1997, and Wainberg and Friedland 1998). For some of these, transmission arises because of incomplete viral suppression (Little et al, 2002). Drug resistant strains that are transmitted may have a higher replication fitness or capacity that enables them to dominate during transmission (Simon et al, 2003). M184V is a marker mutation for transmitted resistance in drug naïve patients in this study and the prevalence for transmitted resistance was 2.9%.

In some acute HIV infections, M184V has been shown to persist in the absence of drugs for a period of a year and over, after acquisition of the infection (Barbour et al., 2004). For patients in our study, transmitted resistance is still low due to a number of reasons. First of all is the introduction of HAART which suppresses or decreases HIV transmission especially when viral load is low (Quinn et al, 2000). In some cases the mutations that develop on the genome of the resistant virus may lower its replication fitness so that the wildtype virus is able to dominate. Studies have shown that transmitted resistant viruses can partially revert to wild type virus resulting into mixtures of wild type and resistant virus (Little et al, 2004). In some cases there may be complete reversion to wild type which however when drugs are introduced, the mutant virus reappears again (Wegner et al, 2000 and Garcia et al, 2001). Due to transmitted resistance, drug naïve patients may poorly respond to ART (Blower et al, 2003). These patients will most likely develop more resistance and this will require more expensive drug regimen to combat the infection. There was no transmitted resistance to PIs mainly because there was low use of

PIs in the population and or because the PI mutations that develop greatly decrease the replication capacity of the virus (Barbour et al, 2002).

The results show that there is some transmission of virus resistant to reverse transcriptase inhibitors and this may result into poor treatment outcomes. In naïve patients, it may not be possible to associate subtypes to drug resistance for reverse transcriptase inhibitors. However for protease inhibitors, resistance is more likely to develop in subtype A than D basing on the numerous minor polymorphisms in the protease gene of subtype A strains.

5.2 Limitations to the study

There were a number of samples whose HIV DNA was not successfully amplified by PCR, and this limited the sample size that was used in the analysis of samples. Unsuccessful amplification could be due to a number of reasons such as, but not limited to Low viral load of the samples and primers used in the amplification being incompatible to the sample HIV DNA. Low sample size may affect the resultant prevalence of drug resistance value as well as the conclusive role of subtypes in HIV drug resistance.

CHAPTER SIX: Recommendations:

High prevalence of RTI resistance as seen in these samples could lead to increased treatment failure especially in settings where reverse transcriptase inhibitors are heavily used (Richards et al, 2004). Drug resistance patterns are expected to change whenever there is increased use of ARVs. In Europe and other developed countries, resistance to ARVs is high because of this increased use of ARVs. This means that the prevalence of resistance is expected to increase as ART becomes available to more patients to sub-Saharan Africa. This will even in turn lead to increased levels of transmitted resistance. The European guidelines 2007 (www.eacs.eu/guide/index.htm), recommend resistance testing for all drug naïve patients, and if resistance testing is not available, then a PI should be included in the first line treatment. However in most settings in the developing world, resistance testing is not readily available. Therefore this calls for regular drug resistance surveillance in naïve patients to monitor prevalence of drug resistance.

Resistance to protease inhibitors is still very low but more phenotypic studies need to be done to confirm that resistance to PIs in experienced patients can be associated to subtypes. Our study shows that resistance in naïve is higher in subtype A patients and predicts the same results for PI experienced patients. We therefore recommend resistance testing and subtyping before patients start on any treatment that contains a PI. Even with HAART, resistance will inevitably emerge (Richman *et al*, 2004). Some of the major mutations result into a compromised viral replication capacity for the virus as well as the virus being resistant to the drugs. The minor resistant mutations will compensate for the compromised viral fitness. The subtype with more minor resistant mutations will therefore be expected to have a better replication capacity in this case. Patients at a high risk for emergence of resistance based on their subtype should then be closely monitored for resistance. Genotypic studies may not alone comfortably answer the question of subtype and their impact on drug resistance or treatment. A combination of phenotypic and genotypic studies will give us a better answer. For genotypic studies, some of the resistant quasi species may not be readily detected and as such only the dominant species is revealed.

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Appendix

April 26, 2008

Chairman
Medical School Institution Review Board,
School of Medicine,
Makerere University

RE: **Permission to Use Samples by the Msc Student**

Dear Dr. Chairman,

This letter to inform the medical school IRB for students that Fred Kyeyune, has been granted permission to use samples from the Genital Shedding study for his Msc research. Fred is a graduate student pursuing his MSc Pharmacology, School of Medicine, Makerere University. We believe that this will be a good opportunity for the student to develop his career in research. In addition the findings from his study will help address some of the unanswered questions pertaining to antiretroviral therapy especially in this era of antiretroviral rollout in Uganda.

It is important to note that the research project described by Fred Kyeyune is part of a larger project entitled "*HIV-1 antiviral outcomes and resistance in African men and women in Uganda and Zimbabwe*" by the JCRC IRB and by UNST in Uganda and by the IRB at University Hospitals of Cleveland. All samples have been obtained under informed consent.

Please contact us with any further questions.

Sincerely,

Robert A. Salata, M.D.

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