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HIV-1 Drug Resistance to Integrase Strand Transfer Inhibitors in HIV-1 non-B Subtypes

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Abstract

Human immunodeficiency syndrome (HIV-1) has infected over 75 million people and over 35 million have succumbed to virus related illnesses. Despite access to a variety of antiretroviral therapy (ART) options, ART programs have been disproportionally spread in the world with low- and middle-income countries (LMICs) facing challenges to access the most potent ART options. With less potent ART remaining in use in LMICs, HIV-1 drug resistance (HIVDR) presents a growing challenge in LMICs. Since approval of the first-generation integrase strand transfer inhibitor (INSTIs), Raltegravir (RAL) in 2007, INSTIs remain the best choice as a backbone of ART. Access to second generation INSTIs, Dolutegravir (DTG) and bictegravir (BIC) in LMICs is based on need and not on a full evaluation of the effectiveness of these treatments in patients infected with non-B HIV-1 subtypes. To address this challenge of limited INSTIs associated HIVDR data in non-B HIV subtypes, we first screened for the presence of INSTIs associated drug resistance mutations (DRMs) in ART naïve and experienced patients in Uganda using Sanger and Illumina sequencing. In Uganda, 47% of patients failing on RAL carry resistance to RAL-and elvitegravir (EVG), and only 4% harbor resistant virus to DTG. A panel of recombinant viruses from patient-derived HIV-1 integrases carrying resistant mutations was created and tested for susceptibility to a panel of INSTIs: EVG, RAL, DTG, BIC, and CAB. The virus carrying N155H or Y143R/S was susceptible to DTG, BIC, and CAB but highly resistant to RAL and EVG (>50-fold change). Two patients, one with E138A/G140A/Q148R/G163R and one with E138K/G140A/S147G/Q148K, displayed the highest reported resistance to RAL, EVG (FC, >1000) and even DTG (FC, >100), BIC (FC, 60->100), and CAB (FC, 429->1000). All viruses had impaired replication fitness and <50% reduction in integration capacity. We further determined potential novel polymorphisms associated with INSTI resistance in HIV-1 subtype A and D using simple vector machine analysis. The identified I208L and I203M, did not show reduced susceptibility to RAL or DTG with 1.3-1.8-fold and 1-1.4-fold observed, respectively. Further investigation is required to determine how these novel mutations influence susceptibility to INSTIs in HIV-1 subtype A and D infected patients.

Keywords

HIV-1 non-subtype B, integrase strand transfer inhibitors, HIV-1 drug resistance, Low-and Middle-income countries, Uganda

Summary for Lay Audience

Human immunodeficiency syndrome virus type 1 (HIV-1) is the causative agent for Acquired immunodeficiency syndrome (AIDS) which is characterized by rapid depletion of $CD4^+$ T cells which eventually results in emergence of opportunistic infections. HIV-1 like other retroviruses, hijack host DNA machinery to be able to replicate and cause havoc in the infected individual. Because of high replication rate and numerous errors during replication, there are diverse strains of HIV-1 circulating in world population which are commonly referred to as subtypes, circulating recombinant forms, and unique recombinant forms. The non-B HIV-1 subtypes are predominantly in low- and middle-income countries (LMICs) and account for up to ~ 90% of HIV-1 infections. The antiretroviral therapy (ART) has tremendously improved the lives of individuals infected with HIV-1 and discovery of an even more potent class of ART, the integrase strand transfer inhibitors (INSTIs) has further improved HIV-1 treatment outcomes. Despite these advances, research of HIV-1 drug resistance associated with INSTIs has historically been done in HIV-1 subtype B virus which is predominant in high-income countries. With improved access to INSTIs by HIV-1 patients in LMICs, there is urgent need to assess susceptibility of HIV-1 non-B strains to INSTIs and also discover novel potential pathways to resistance in these strains. From a cohort of HIV-1 infected patients in Uganda, we show that prolonged virological failure on INSTI, raltegravir, leads to accumulation of drug resistance mutations which confer resistance to all currently available INSTIs. Novel mutations in the HIV-1 integrase gene which show no impact on INSTIs susceptibility by themselves, may act as secondary mutations to other drug resistance mutations outside HIV-1 integrase gene which merits further investigation.

Co-Authorship Statement

I am the sole lead author of the first three manuscripts presented in this work. Though I am the second author in the fourth manuscript, I carried out much of the work presented including, sample collection, laboratory assays, participated in data analysis, and writing of the manuscript. Other co-authors of the manuscript each had a role in the study including designing of experiments, recruitment of patients, data analysis, writing and providing critical reviews of the manuscript.

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List of abbreviations

3TC	lamivudine
AIDS	Acquired Immunodeficiency Syndrome
APOBEC3G	Apolipoprotein B MRNA Editing Enzyme Catalytic subunit 3G
ART	Antiretroviral Therapy
ATV	atazanavir
AZT	zidovudine
BAF	barrier-to-autointegration factor
BIC	bictegravir
CA	capsid
CAB	cabotegravir
cART	combined antiretroviral therapy
CCD	catalytic core domain
CCR5	C-C chemokine receptor type 5
cDNA	complementary DNA
CRF	circulating recombinant form
CTD	C-terminal domain
CXCR4	CXC chemokine receptor type 4
DDB	DNA binding protein
DNA	deoxyribonucleic acid
DNA-PK	DNA protein kinase
dNTPs	deoxyribonucleotide triphosphate
DRMs	drug resistance mutations
DRV	darunavir
DTG	dolutegravir
EFV	efavirenz
ETR	etravirine
EVG	elvitegravir
FDA	Food and Drug Administration
FTC	emtricitabine
HAART	highly active antiretroviral therapy
HIV-1	Human Immunodeficiency Virus type 1

HIVDR	HIV-1 drug resistance
HIV-1 IN	HIV-1 integrase
HMGA1	high mobility group protein A1
HR	heptad repeat
HRP-2	hepatoma-derived growth factor related protein 2
HSP	heat shock protein
IAS	international AIDS society
IN	integrase
IN-1	integrase interactor 1
JCRC	Joint Clinical Research Center
LAP2alpha	lamina-associated polypeptide 2 alpha
LEDGF/p75	lens epithelium-derived growth factor/transcription coactivator
LTR	long terminal repeat
MA	matrix
mRNA	messenger RNA
NC	nucleocapsid
NEF	negative factor
NHEJ	non-homologous end joining
NLS	nuclear localisation signal
NNRTI	non-nucleoside reverse transcriptase inhibitors
NRTI	nucleoside reverse transcriptase inhibitors
NTD	N-terminal domain
NVP	nevirapine
PBMCs	peripheral blood mononuclear cells
PDR	pretreatment drug resistance
PEPFAR	President's Emergence Plan for AIDS Relief
PIC	preintegration complex
PPT	polypurine tract
P-TEFb	positive transcription elongation facotr
RAL	raltegravir
RC	replication capacity
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
RPV	rilpivirine

RRE	rev response element
RSV	respiratory syncytial virus
RT	reverse transcriptase
SIV	Simian Immunodeficiency Virus
TAF	tenofovir alafenamide
TAR	trans-activator response
TAT	trans-activator of transcription
TDF	tenofovir alafenamide
TFIID/TFIIA	transcription factor II D/Transcription factor II A
TRIPS	Trade-related aspects of intellectual property rights
UNAIDS	Joint united nations programme on HIV/AIDS
URF	unique recombinant forms
vDNA	viral DNA
VIF	viral infectivity factor
VPR	viral protein R
VPU	viral protein U
vRNA	viral RNA
WT	wild type

Chapter 1

1 Introduction

1.1 HIV-1

The human immunodeficiency virus type 1 (HIV-1) belongs to a subgroup of retroviruses which infects humans and was first recognized in 1981 among a group of homosexual mean in the US (1,2). In 1982, a resident doctor in the East African country of Uganda started seeing patients with major clinical manifestations of severe weight loss and diarrhea among heterosexual individuals (3). This was a kick start for global awareness campaigns of a strange new disease called GRID (gay-related immunodeficiency disease) in the US and SLIM disease in East Africa (Slim being related to emaciation of immunodeficient patients). HIV-1 was later discovered (1983) as the causative agent for Acquired Immunodeficiency syndrome (AIDS) (formerly GRID and SLIM) (4–6) which is characterized by severe depletion of $CD4^+$ T cells leading to susceptibility to numerous opportunistic infections and cancers (7–9). If left untreated, the average time of survival is about 9-10 years depending on the virulence of infecting HIV-1 strain. The current literature indicates that HIV-1 was introduced into humans between 1920-1940 (10). Based on phylogenetic analysis, HIV-1 is classified as HIV-1 based on the zoonotic transmission to humans from simian immunodeficiency virus (SIV) from chimpanzee (SIVcpz) and gorilla (SIVgor) in West Central Africa, and HIV-2, based on the transmission from West Africa sooty mangabeys (*Cercocebus atys*, SIVsmm) (11,12). HIV-1 was first isolated in 1983 and HIV-2 in 1986, and both spread and diversified exponentially to create numerous subtypes that are circulating today.

1.2 HIV-1 particle and genome

HIV-1 is roughly spherical with a diameter of ~100nm-150nm and two identical single strands of RNA enclosed in the viral core (Fig. 1). HIV-1 has a total of 15 proteins from three major open reading frames, *gag*, *pol*, and *envelope*. The outer membrane proteins are translated from *env* open reading frame and they include highly glycosylated surface protein (SU, gp120) and transmembrane (TM, gp41) glycoproteins for attachment and fusing to host cell. From outside to inside, there is outer core membrane matrix (p17, MA) protein, capsid (p24, CA) protein and

nucleocapsid (p7, NC) protein that is tightly bound to genomic RNA strands (Fig. 1). Functional viral enzymes are coded by the *pol* open reading frame and they include, protease (p12, PR), reverse transcriptase (p66, RT), RNase H (the C-terminal p15 of RT p66) and integrase (p32, IN). Rev (RNA splicing regulator), and Tat (transactivator protein), act as regulatory proteins controlling RNA transcription and metabolism. Accessory genes, Vpu (viral protein unique), Vif (viral infectivity factor), Vpr (viral protein r), and Nef (negative regulatory factor), play critical roles in viral pathogenesis, replication and budding of the virus among a host of other regulatory functions during HIV-1 lifecycle or to avoid host restriction factors (13,14). The genomic RNA is flanked on both ends by identical long terminal repeats (LTRs) sequences with 5' LTR acting as promoter site and 3′ LTR having polyadenylation signal. HIV-1 and HIV-2 have similar genome structure except that Vpx is encoded in HIV-2 in place of Vpu found in HIV-1 (15).

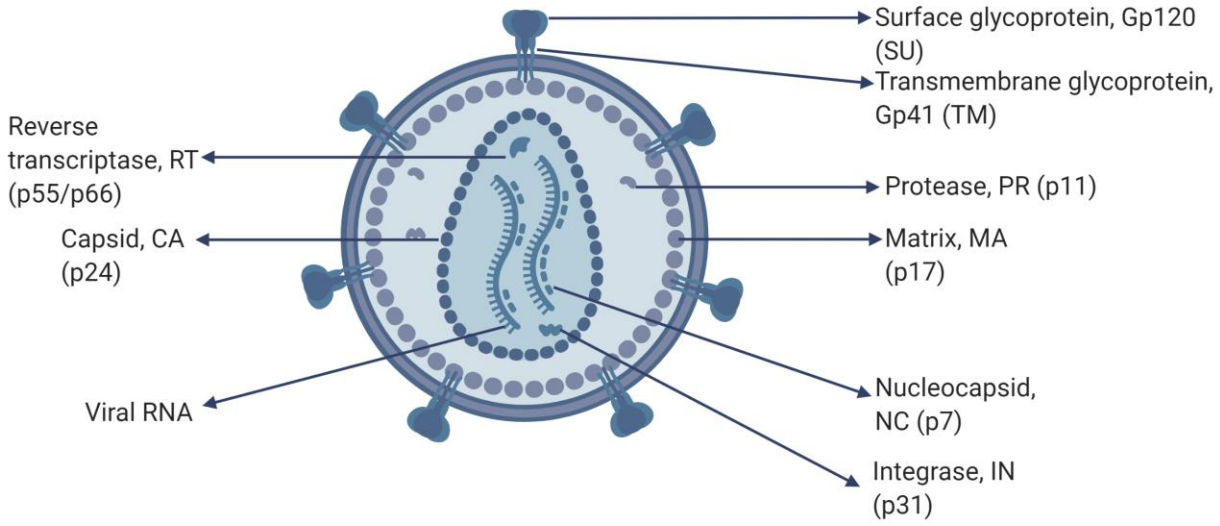

Figure 1. HIV-1 particle. At the center of HIV-1 mature virion, there are two copies of viral RNA tightly associated with nucleocapsid protein and copies of reverse transcriptase and integrase viral enzymes. These are encapsulated in cone-shaped capsid protein which is also surrounded by protease enzyme and matrix protein. The outer membrane consists of host-derived cell membrane and includes transmembrane glycoprotein (gp41) which anchors surface glycoprotein (gp120).

1.2.1 Structural proteins

Gag is expressed in the form of a 55-kDa precursor, $Pr55^{Gag}$ and is the major structural component of HIV-1. The $Pr55^{Gag}$ polyprotein is cleaved by HIV-1 protease to structural proteins p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), spacer protein1, spacer protein 2, and p6 proteins (16,17) (Fig. 2).

CA (p24) is a cone-shaped protein which surrounds the two strands of viral genome and viral proteins. It is mainly involved in maturation of virions and virus assembly processes. CA is made of two domains, N-terminal core domain (NTD, 1-145) and C-terminal dimerization domain (CTD, 151-231). The NTD is highly helical due to secondary structure of 7 alpha helices and 2 β-hairpin, in addition to exposed loop which acts as binding site for cyclophilin A (18). Whereas, NTD is implicated in maturation of virions and incorporation of cyclophilin A into the virion, CTD has been shown to be involved in recruitment of Gag-pol ($Pr160^{gag\text{-}pol}$) precursor which eventually facilitate incorporation of viral proteins IN, RT and PR (19). The CTD is also involved in Gag oligomerization (20,21). Despite NTD being a core domain for CA, induced mutations in this domain generally have no effect on HIV-1 virion production, whereas mutations in CTD cause significant loss of virion particle assembly and release by proviral DNAs (22).

NC (p7) is typically bound to genomic RNA in the HIV-1 particle core. It contains two zinc finger-like motifs (Cysteine-Histidine boxes) which are highly conserved in retroviruses and each box coordinates a zinc ion. p7 is involved in packaging of genomic RNA and segments of the protein may facilitate assembly and release of viral capsid particles (23).

MA (p17) is a protein of 128 amino acids made up of 5-α helices and mixed β-sheet. It forms the N-terminal end of $Pr55^{Gag}$ precursor protein. MA forms a core domain of mainly α -helices and three-stranded β-sheet while the C-terminus of the protein comprises an α-helix that connects MA and CA. The protein plays a critical role in intracellular targeting of polyproteins (24) including targeting Gag precursor to the site of viral assembly on the plasma membrane, and incorporation of Env glycoprotein into the virions. MA also targets the preintegration complex (PIC) to the nucleus by providing a nuclear localisation signal.

HIV-1 p1 also known as spacer peptide 2 (sp2) is a protein derived from cleavage of $Pr55^{Gag}$ and is located at C-terminus of Gag between NC and p6 (Fig. 2). It is made up of 16 amino acids with two highly conserved proline residues, Pro-439 and Pro-445 and substitution to leucine decreases infectivity and reduces stability of RNA (25). It is crucial for Gag and Pol incorporation into virus particles (26).

HIV-1 p2, also known as spacer peptide 1 (sp1), is a 14 amino acid peptide that is found between the CTD of CA and NTD of NC proteins (Fig. 2). p2 plays a significant role in CA assembly and HIV-1 infectivity (27–29). Deletion of p2 results in decreased ordered assembly and infectivity which results in "bent" core structures in virus particles produced. (27–29). Gag constructs lacking p2 are only able to form tubular and conical shapes but not spherical viral particles (30).

Ribonucleoprotein formation of genomic RNA and condensation of CA core has been shown to be influenced by proteolytic processing of p2 from NC. CA-p2 cleavage during late viral processing enables CA shape by modulating CA-CA interactions.

HIV-1 p6 is a 52 amino acid protein that is important in formation of infectious viruses and could be categorized as regulatory versus structural protein. P6 aids the process of HIV-1 particle maturation by incorporation of Vpr into virus particles (31) and supports budding off of HIV-1 virion from host cell membrane (32). It has been shown to interact with Vpr and cyclophilin A (33). HIV-1 p6 is also involved in regulation of CA processing and virus core (34), and MHC-class 1 presentation of Gag (35).

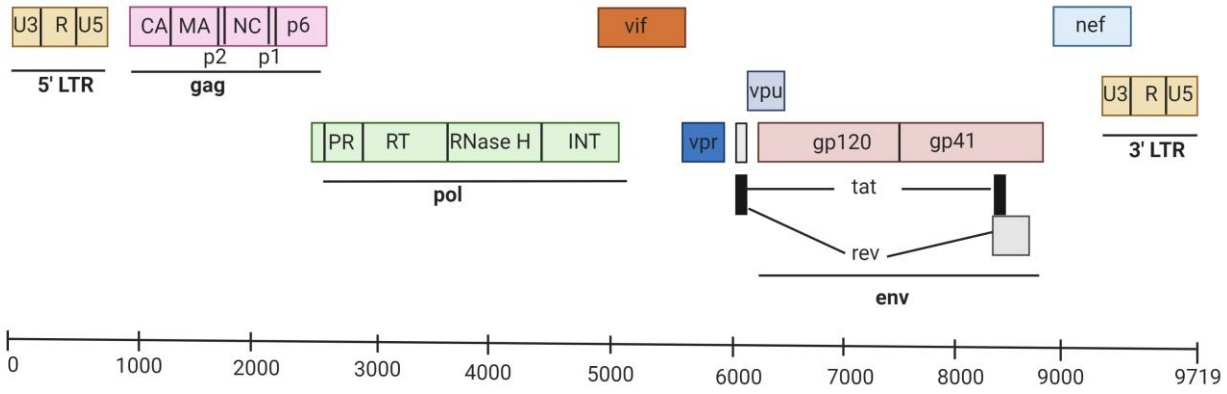

Figure 2. HIV-1 genome structural organization. HIV-1 is made up of 9719 base pairs in total with three major open reading frames of *gag, pol*, and *env*. HIV-1 genome is franked with similar sequences in 5′ long terminal repeat and 3′ long terminal repeat regions which are important for regulatory functions such as transcription and polyadenylation.

Pol: The proteolytic processing of $Pr160^{gag\text{-}pol}$ polyprotein by HIV-1 protease yields structural proteins, MA, CA and Pol and catalytic enzymes RT, PR, RNase H, and IN (36,37) (Fig. 2 and 3). Gag-Pol precursor is a product of fusion of Gag and Pol polyproteins from -1 translational frameshift which happens at UUA leucine codon near 5′ end of overlap region between Gag and Pol. The ribosomal frameshift signal consists of "slippery" sequence U UUU UUA and upstream ACAA tetraloop and this ensures 5% of -1 Pol open reading frame and 95% Gag alone products (38,39).

Reverse transcriptase: The mature form of HIV-1-RT is an asymmetric heterodimer enzyme containing a 66 and 51 kDa protein produced and possibly self assembled following the cleavage of gag-pol polyprotein by HIV-1 protease during virion maturation. In each HIV-1 particle, there are about 50 p66/p51 RT molecules (40). Only one (p66) of two subunits contains a functional polymerase and RNase H domains (41). Despite sequence homology between the p66 and p51 subunits, polymerase has a different structure between the two subdomains with p66 forming a large active-site cleft and p51 exhibiting an inactive closed morphology. The p66 polymerase active site has a catalytic amino acid triad (Asp 110, Asp 185 and Asp 186) in the "palm" subdomain, which is similar and conserved in many reverse transcriptase enzymes and other RNA polymerases (41–43). When RT binds to a primer-template nucleic acid duplex the catalytic triad in palm is positioned "over" the 3′-OH of the primer terminus and catalyzes the nucleophilic attack on the incoming nucleoside triphosphate releasing a diphosphate and forming a covalent bond with

primer terminus and this new nucleoside monophosphate. As such p66 is involved in catalytic activities and p51 in structural roles of HIV-1-RT (41,43). HIV-1 RT catalyses reverse transcription of genomic RNA. The C-terminus of the p66 subunit of RT forms RNase H domain (441-560) which degrades the RNA genome except for the PPTs (44). The full process of HIV-1 reverse transcription is described in section 1.3.3.

Protease is a 99 amino acid aspartyl protease which cleaves the Gag and Gag-Pol precursors during viral assembly to form structural and functional enzymes (45) (Fig. 3). The initial processing is initiated by activated protease releasing itself from Gag-Pol precursor (46). It then cleaves Gag-pol and Gag polyproteins into functional enzymes, RT, PR, IN and structural proteins, matrix (MA), capsid (CA), nucleocapsid (NC), and proteins p1, p2 and p6 (45). It is only functional as a dimer with only one active site which consists of two identical subunits with a two-fold (C2) symmetry.

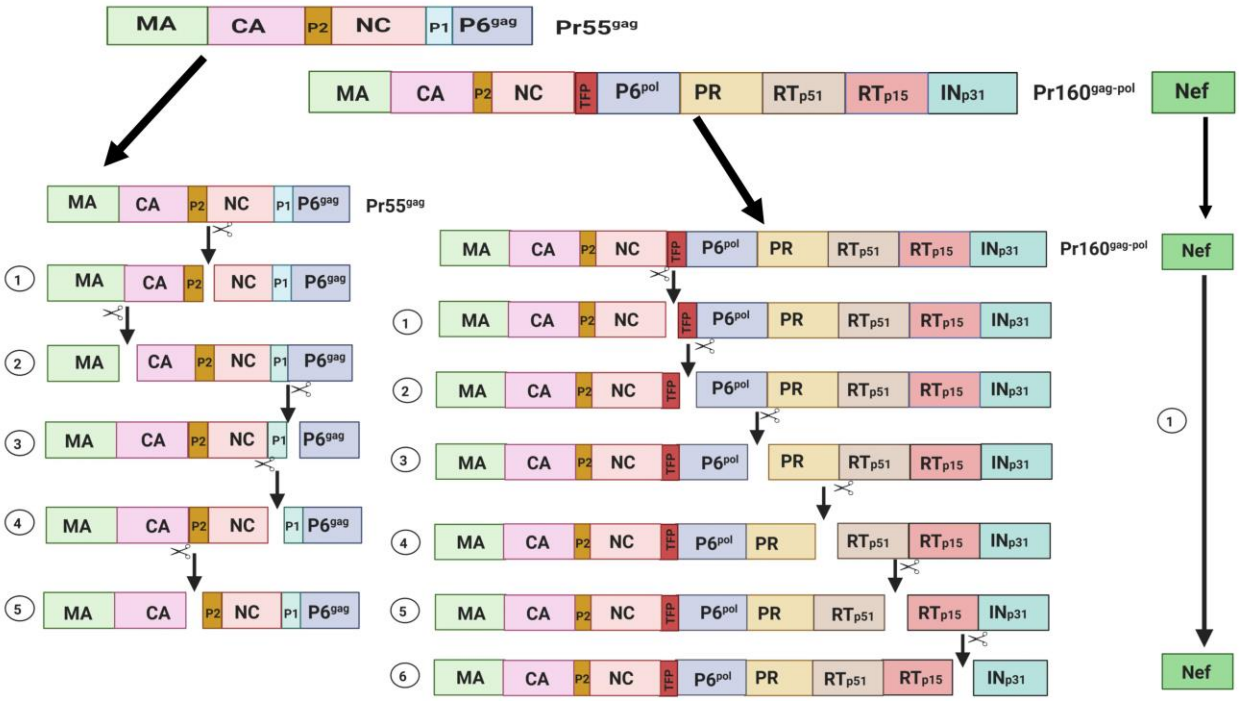

Figure 3. HIV-1 Pr55^{Gag} and Pr160$^{gag\text{-}pol}$ polyprotein cleavage by HIV-1 PR. The polyproteins Pr55^{Gag} and Pr160$^{gag\text{-}pol}$ from transcription of provirus are cleaved by HIV-1 PR enzyme late during assembly and maturation of new virions. Pr55^{Gag} is proteolytically cleaved by HIV-1 PR in orderly manner with first cleavage happening between p2 and NC followed by MA and CA then p1 and p6, NC and p1, and finally CA gets cleaved from p2. Pr160$^{gag\text{-}pol}$ polyprotein from ribosomal frameshift contains region with transframe locus which encodes p6 and a transframe protein covalently linked to N-terminus of HIV-1 PR. Pr160$^{gag\text{-}pol}$ gets cleaved into gag structural proteins of MA, CA, P2, NC, P6 and pol proteins, PR, RT_{p51}, RT_{p15} and IN.

Integrase: HIV-1 integrase (HIV-1 IN) is a 32 kDa protein produced from gag-pol polyprotein cleavage by HIV-1 protease gene and about 40-100 integrase molecules are packaged in each HIV-1 virion. HIV-1 IN is involved in a number of viral steps of reverse transcription (47), nuclear import of PIC (48,49), and integration of cDNA into host chromosome (50). The process of HIV-1 integration into host chromatin is a two-step process of 3' processing and subsequent strand transfer process both mediated by HIV-1 IN. In 3' processing, HIV-1 IN dimer binds to each end of the newly synthesized linear viral DNA (vDNA) and cleaves the two nucleotides (GT) from each 3′ end which leaves conserved CA dinucleotide at terminus and exposes hydroxyl group (CA_{OH}) (51,52). The cleaved vDNA is a component of the PIC that translocates into the nucleus for subsequent strand transfer process. Details of this HIV-1 integrations process are described in sections 1.3.4, 1.3.5 and 1.3.6.

HIV-1 IN is composed of 288 amino acids and 3 domains, NTD (NH_2-) (1-49), catalytic core domain (50-212)-CCD, and CTD (213-288) (COOH-) (53) (Fig. 4). HIV-1 IN is relatively conserved compared to RT and PR HIV-1 enzymes, with well-defined cytotoxic T lymphocyte epitopes of n=11, compared to PR, n=7 and RT, n=41 (54). All the three domains of HIV-1 IN are required for 3′ processing and strand transfer processes (55,56). However, disintegration can solely be carried out by CCD (56,57). When strand transfer product is added to a protein mixture of mutated NTD and mutated CTD both with active site, it was shown that 3′ processing and strand transfer only occur after complementation with a protein with active site mutants which shows that HIV-1 IN functions as a multimer (58). In solution, HIV-1 IN forms dimers (59–62) and tetramers (61,62), and each domain taken separately can also form dimers: the same CCD+CTD and CCD+NTD. The active form of HIV-1 IN during integration is shown to be a tetramer upon interaction with the 3′ and 5′ ends of vDNA (63,64) (Fig. 4).

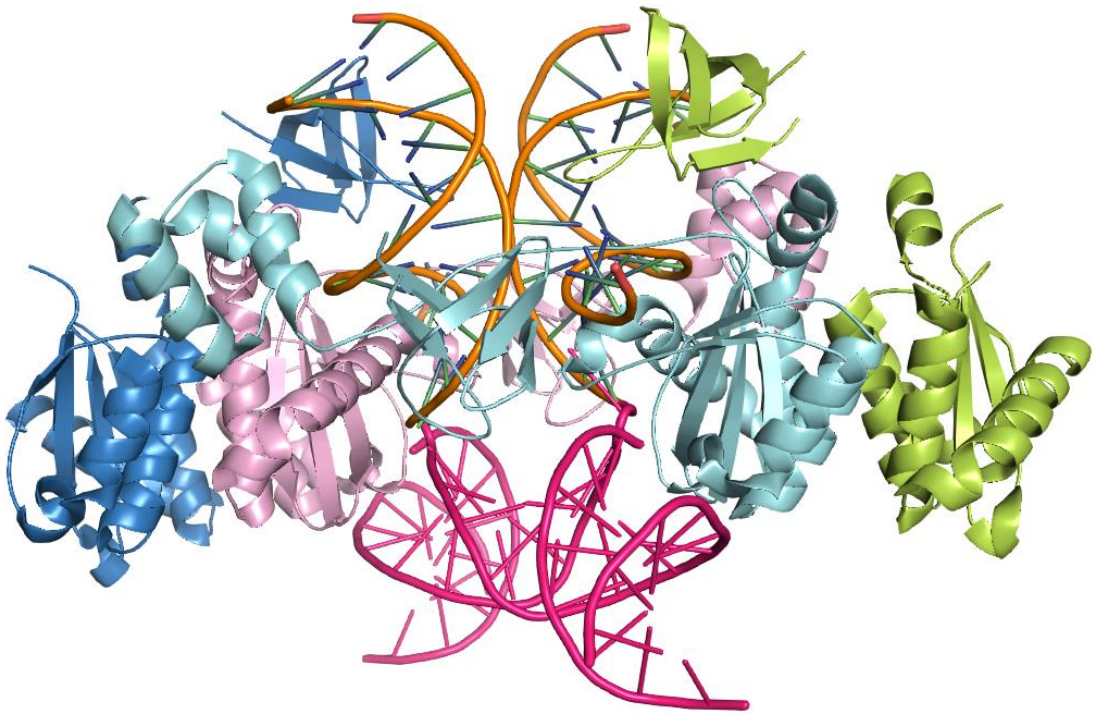

Figure 4. The intasome complex of HIV-1 IN, vDNA and target DNA. The active form of HIV-1 IN when bound to 5′ and 3′ ends of vDNA is a tetramer as shown by four HIV-1 IN monomers colored (sky blue, limon, cyan, and pink). The vDNA is depicted in orange and target DNA, hot pink. The image was generated using Pymol from cryo-electron microscopy structure (PDB ID, 5U1C) (65).

The NTD structure has been solved for both HIV-1 and HIV-2 using nuclear magnetic resonance spectroscopy and it shows a domain with a bundle of 3 α-helices that are stabilised by Zn^{2+} (66,67). The isolated NTD is folded and only becomes a dimer when zinc ions are present (66,67). The NTD of HIV-1 like all other retroviruses has a pair of highly conserved His and Cys residues (HH-CC) that forms a zinc finger (68). Despite having a different structure than the canonical zinc fingers, the HH-CC motif of HIV-1 IN binds zinc (69,70) which allows NTD folding, multimerization, and enhancement of enzymatic catalytic activity (70). There is less effect on disintegration and impairment of 3′ processing and DNA joining by mutations induced in HH-CC motif of NTD (60,71–74). Mutations in HH-CC motif reduce ability of HIV-1 IN to bind zinc (57). However, integrase lacking HH-CC motif are still able to recognise the CA/GT at the end of viral DNA but cannot catalyze the 3′ processing activity (60).

The crystal structure of CCD shows a α/β structure consisting of a 5-stranded β-sheet and 6-α helices numbered 1-6 (Fig. 5). The α4 contains residues Y143 and Q148 which directly bind terminal ends of vDNA (75). Between α5 and α6, there is a flexible finger-like loop of glycine rich sequence of about 12 residues (F185-A196) in HIV-1 (Fig. 5). The loop is stabilised by connections for example a salt bridge between R187 and E198 in HIV-1 has been shown to support enzymatic activity, conformational structure rearrangement of IN on binding DNA, nuclear localisation, stabilising tetramerization, and viral infectivity. The CCD domain is relatively resistant to proteolysis (71) and binds ends of vDNA via residue Q148. The Q148 lies within a flexible loop of residues ^{140}GIPYNPQSQG^{149} that is disordered in most integrase crystal structures but suggested to become ordered upon DNA binding and is involved in stabilising the 5′-end of vDNA (75,76). The interaction between Q148 residue and 5′-C at the end of vDNA enables efficient strand-transfer process by HIV-1 IN (77). The domain has a highly conserved motif of acidic residues, D64, D116, and E152 (D,D-35-E) motif present in all retroviral integrases and transposases. This motif embedded in a RNase H fold protein is used to bind metallic cationic cofactors in all retroviruses, retrotransposon integrase proteins, and some prokaryotic transposases (71,74,78,79), and they are in a similar position in ribonuclease H (76). The D,D-35-E enables enzymatic activity by binding to at least one divalent metal cofactor Mg^{2+} or Mn^{2+} (80–83) and is also implicated in nuclear import (48,84). Within the CCD, the active site is contained within amino acids 50-212, as evident from recombinant peptide encoding this segment being sufficient to promote disintegration and introducing even conservative mutations at any of the catalytic D64,

D116, and E152 residues results in abrogation of catalytic activity (71,73,74). Despite CCD containing the catalytic site for the enzyme, NTD and CTD are indispensable for 3′ processing and strand transfer processes (85). In CCD, a 13-amino acid sequence ^{161}IIGQVRDQAEHLK^{173} located outside the catalytic triad motif but around a surface exposed loop connecting α4 and α5 helices has been shown to have karyophilic property (86).

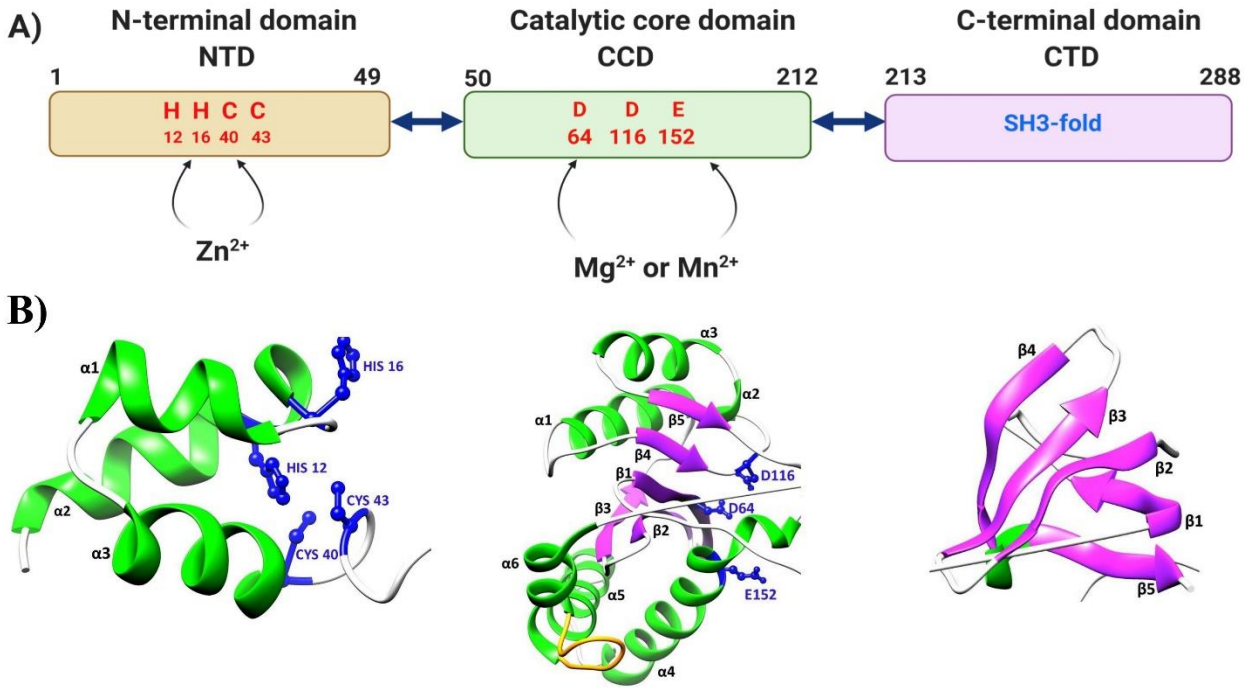

Figure 5. The structural organization of HIV-1 integrase. A) The N-terminal domain consists of 3α-helices with a zinc finger-like motif of 2-His and 2-Cys amino acids for coordinating zinc ions. The catalytic core domain is made up of 6 α-helices and 5-stranded β-sheet with highly conserved residues D16, D116 and E152 for coordinating magnesium ions required for catalytic activity of the enzyme. The C-terminal domain is least conserved with an SH3-fold structure. B) The crystal structure of HIV-1 IN for antiretroviral therapy naïve patient sample 'GS221' was predicted using template d1wfa (99.9% confidence) for NTD, d1c6vx (100% confidence) for CTD and d1hyva (100% confidence) for CCD in PHYR2 (87). The α-helices are shown in green, β strands in magenta, the finger-like loop between α5 and α6 of CCD is shown in orange and divalent metal coordinating residues of NTD and CCD shown in blue. The structures were visualized and analyzed in UCSF Chimera (88).

The CTD is least conserved among all retroviral integrases and binds DNA non-specifically. Based on NMR spectroscopy, the solution structure of CTD domain forms a homodimer of five β-strands

which make two antiparallel β sheets (68,89). The overall folding topology of CTD is closely similar to that of Src-homology 3 (SH3-fold) (68,89,90); though there have been variations in CTD interfaces in IN crystal structures containing CTD domain (91). When CTD is joined to CCD, it forms a monomeric structure (91). The structure of full-length HIV-1 IN with all three domains has been elusive due to the natural tendency to form large aggregates under test conditions. But the structure of double domains of CTD/CCD for HIV-1, SIV and RSV (91–93), and NTD/CCD has been solved. Sequence fragment of minimal region of CTD (220-270) is adequate for DNA binding (94) and residues between 245-270 make interactions with bases A (4) and T (5) of vDNA end (75,95). Mutation of CTD basic residue K264 in HIV-1 results in a significant decrease in DNA binding and catalytic activity (94). Mutating residues Leu 241 and Leu 242 to alanine impairs dimerization which significantly decrease catalysis activity (96). CTD may be critical in stabilising interaction of HIV-1 IN and vDNA by aligning DNA to fit in position that allows proper binding of viral terminal ends. In support of this observation, CTD is involved in 3' processing but not in disintegration process (97). CTD also provides a karyophilic property of IN by having a bipartite NLS of amino acids ^{211}KELQKQITK^{219} and ^{261}PRRKAK^{266} which are recognised by karyopherin-α (48). To function properly, HIV-1 IN associates with a number of cellular proteins which act as catalytic cofactors for integration processes. High mobility group protein A1 (HMGA1) and barrier to autointegration factor (BAF) appear to directly interact with DNA substrate whereas, lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75), EED and HSP have been shown to directly interact with HIV-1 IN.

Env: HIV-1 env gene is about 2.5 kb and is translated into 850 amino acids. HIV-1 *env* is first produced as precursor glycoprotein gp160 that undergoes a number of stages during its maturation including, formation of disulfide bonds, extensive glycosylation, cleavage by furin-like proteases, transport to cell membrane, and package into HIV-1 particle (98). Gp160 undergoes glycosylation in the endoplasmic reticulum and subsequently gets cleaved by cellular furin-protease in golgi apparatus, producing surface glycoprotein gp120 (~ 480 amino acids) and transmembrane glycoprotein gp41 (~ 345 amino acids). Gp120 has five conserved domains (C1, C2, C3, C4 and C5) and five variable domains (V1, V2, V3, V4, and V5). HIV-1 gp41 consists of seven domains, N-terminal fusion peptide, heptad repeat 1 (HR1), disulfide loop, HR2, membrane proximal ectodomain region, transmembrane and cytoplasmic tail (99). A trimer of heterodimers from three gp120s and gp41s form envelope spike protein (100). During HIV-1 entry, gp120 of envelope

spike protein that is ~ 50% carbohydrate, recognises and binds to primary receptor CD4 (101) which induces conformational changes in gp120 and allows recognition of C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) (102). This triggers conformational change of gp41 which triggers heptad repeats HR1 and HR2 to form a 6-helical bundle which creates a pore to allow fusion with host cell plasma membrane and delivery of viral genome into the cytoplasm of host cell (103).

1.2.2 Accessory proteins

HIV-1 Nef: It is a multifunctional 27-kd myristoylated protein that is encoded by a single exon that extends into the 3' LTR of primate retroviruses. After packaging in virions, Nef is cleaved by HIV-1 protease during maturation (104,105). It is the first protein to be produced to sufficient levels inside an infected cell (106). Although Nef is predominantly localised in the cytoplasm and associated with the plasma membrane via the myristoyl residue, it has also been found in the nucleus in some studies. It is implicated in increasing the infectivity of the virus. In early phases of HIV-1 infection, Nef downregulates CD4 from the cell surface by endocytic pathways involving clathrin and AP2 (107–109) and eventually degraded in lysosomes (110,111). Downregulation of CD4 is crucial because accumulation of CD4 on cell surface prevents Env incorporation and budding (112,113). Nef is also implicated in downregulation of MHC-1 molecules from cell surface by rapid internalisation and degradation in endosomal vesicles (114), which inhibits lysis by cytotoxic CD8+ T cells and for viral spread and disease progression *in vivo*. Defective Nef has been observed in HIV-1-infected long term progressors, (115) indicating its role in maintaining high viral loads in infected individuals. Nef increases viral infectivity in both lymphocytes and macrophages (116) and relative infectivity of virus particles in Nef (+) compared to Nef depleted virus has been shown to be 3-to10-fold higher (117).

HIV-1 Vpu: viral protein U, is a 16-kDa of 81-amino acid that is expressed late in HIV-1 life cycle and is coded by an open reading frame between the first exon of *tat* and *env* genes (118,119). Vpu is a product of Rev-dependent bicistronic mRNA which also encodes *env* showing their coordination in HIV-1 infection (120). Vpu is unique to HIV-1, and its closest homologs are in SIV from chimpanzee (SIVcpx) and SIV from rhesus mona monkeys (*Cercopithecus mona*;

SIVmon), SIV from greater spot-nosed monkey (*Cercopithecus nictitans*; SIVgsn), SIV from mustached monkey (*Cercopithecus cephus*; SIVmus), SIV from Dent's mona monkey(*Cercopithecus mona denti*; SIVden) and SIV from gorilla (*Cercopithecus Gorilla gorilla*; SIVgor) (121–126). There is no similar protein in either HIV-2 nor other related SIVs, SIV from sooty mangabey (SIVsmm) and SIV from rhesus macaques (SIVmac) (118,119). It is implicated in degradation of CD4 molecules by mediating the proteasomal degradation of newly synthesized CD4 molecules in endoplasmic reticulum (127). It also enhances virion release from plasma membrane of HIV-1 infected cells (119,128) by antagonizing interferon-regulated host restriction factor-tetherin which directly cross links virions on host cell surface (129–131) .

HIV-1 Vpr: the viral protein R (Vpr) is a 14-kDa protein of 96-amino acid that is incorporated into the virion with around 100 copies of Vpr copies present in each virion (132). The incorporation of Vpr in virions is mediated by specific interaction with carboxyl-terminal region of $Pr55^{Gag}$ (133). Vpr is involved in binding of PIC to importins and nucleoporins to promote nuclear import of HIV-1 into non-diving cells (134,135), and activation of HIV-1 LTR through binding to sp1 (136) or primarily through sequences of -278 to -176 of HIV-1 LTR (137). Vpr is also involved in preventing infected cells from passing through mitosis by arresting them in G2 phase (G2 cell arrest) through interactions with a DDB_1 and $Cullin_4A$-containing ubiquitin ligase complex (138–142) and direct and indirect promotion of T-cell dysfunction. Vpr induces T-cell apoptosis through inhibition of NF-kB activity (143), but it is also involved in inducing the NF-kB activity by phosphorylation and degradation of IkB (144). In HIV-2, G2 cell arrest is still carried out by Vpr and protein Vpx (close sequence homology to Vpr) is involved in PIC nuclear import (145,146). Interaction of Vpr with cellular Uracil-DNA glycosylase, a protein implicated in DNA repair process (removes uracil from synthesized DNA) influences *in vivo* mutation rate (147).

HIV-1 Vif: Viral infectivity factor is a 23 kDa protein that is incorporated in virions (148) and exists in both membrane-associated and soluble cytosolic forms. Vif is required during late stage of infection and promotes viral infectivity by 10-1000 fold (149–152). Vif protein also antagonizes the activity of Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 3G (APOBEC3G) enzyme which redirects it by ubiquitination to degradation in the proteasome (153–155) through recruitment of Cullin CUL5, elongins B and C, and Rbx1 to form ubiquitin ligase complex which induces APOBEC3G degradation (156).

1.2.3 Regulatory proteins

HIV-1 Rev: is a 19 kDa protein made up of 116 amino acids that is critical for regulation of HIV-1 protein expression. It is a product of completely spliced mRNA transcript. It functions as a tetramer and a nuclear localisation signal encoded by the protein allows its localisation in the nucleus. Rev sequence has an arginine-rich sequence on amino-terminal domain which acts as a nuclear localisation signal (157–160) and RNA-binding domain (157,161–165). The flanking sequences on both sides are required for multimerization (163–165). The leucine-rich carboxyl terminal domain of Rev (158,164,166) exhibits nuclear export signals (167–169). It is involved in nuclear export of intron-containing ~ 9 kb-unspliced (Gag and pol) and ~ 4-kb incompletely spliced (Env) transcripts into the cytoplasm for translation or packaging into new virions (170–172). HIV-1 Rev response element (RRE) found in Env region of unspliced and partially spliced mRNAs is required for Rev function. Rev in the presence of RRE increases levels of both unspliced (Gag) and partially spliced (Env) mRNAs (173) and decreases the amount of mRNAs targeted for splicing. In early (Rev independent) stage, ~ 2-kb mRNA transcripts which encode Tat, Nef, and Rev proteins accumulate in the cytoplasm, and importation of Rev into the nucleus allows binding to RRE of ~ 9 kb and ~ 4-kb transcripts which enables their nuclear export out of nucleus in late Rev-dependent stage. In absence of Rev, late genes, Gag, Pol, Env, Vpr, Vpu and Vif are localised in the nucleus and cannot be translated.

HIV-1 Tat: Reverse transcription of provirus DNA is regulated by HIV-1 trans activator protein, Tat, which enhances the process of reverse transcription by binding to the *cis*-acting stem loop element in the 5’ end of HIV-1 LTR encompassing nucleotides +1 and +59 known as TAR (174). TAR is made up of the first ~60 nucleotides of nascent RNA transcript 5’ end LTR promoter and acts as a binding site for Tat and assembly site for a large number of transcriptional elongation factors. TAR sequence has a region of highly conserved 3-nucleotide pyrimidine bulge that binds Tat and 6-nucleotide loop for binding positive transcriptional elongation factor P-TEFb (175). The binding of Tat to TAR region, recruits P-TEFb (cyclin T1/ cyclin dependent kinase 9) to TAR region. With Tat bound to TAR, pTEFb recruits TATA box binding protein to the promoter region and CDK9 phosphorylates the C-terminal domain of RNA polymerase II which increases processivity of RNA polymerase. In absence of Tat, transcription initiates but only short transcripts

within TAR (between positions +55 and +59) are produced due to early termination by RNA polymerase II enzyme (176).

1.3 Overview of HIV-1 life cycle

HIV-1 undergoes several steps in a life cycle that only lasts 24-48 hours. Each step is crucial for effective viral replication and this has allowed the discovery of different antiviral compounds which target different stages to halt HIV-1 viral replication (Fig. 6). HIV-1 uses a number of viral and cellular proteins during its replication to produce nine open reading frames which code for 15 proteins. Please note that this review will focus on HIV-1 reverse transcription, protease processing, and integration which are the primary targets for the current and most effective antiretrovirals. Other steps in the lifecycle of HIV-1 are not described in detail.

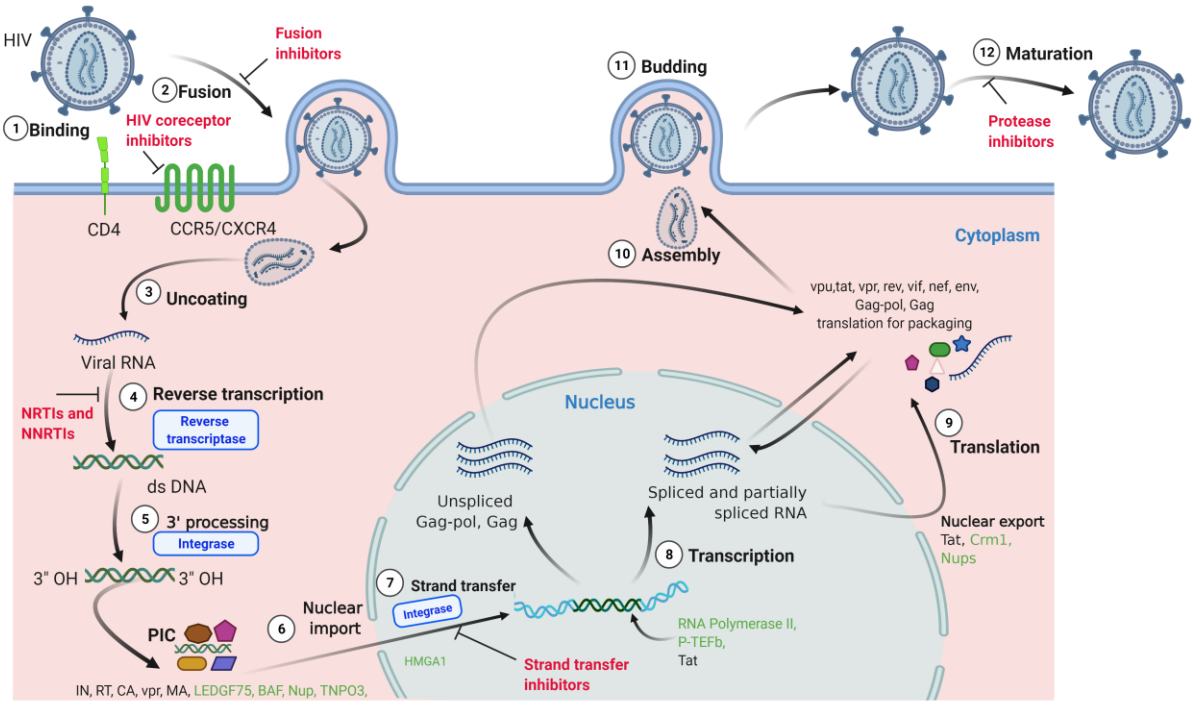

Figure 6. HIV-1 replication life cycle. 1) HIV-1 surface glycoprotein gp120 binds to host cell through mainly CD4+ receptor. 2) Fusion with host cell membrane occurs after a change in conformation of gp120 to expose gp41 transmembrane glycoprotein. 3) HIV-1 core uncoats inside the cytoplasm to release reverse transcription complex where genomic RNA is reverse transcribed.

4) HIV-1 genomic RNA is reverse transcribed by reverse transcriptase enzyme into double stranded DNA. 5) Dinucleotide 'GT' of synthesized viral DNA is cleaved at conserved CA site on U3 and U5 of 3′ end within the preintegration complex (PIC) by HIV-1 IN. 6) The nuclear localization signal of PIC components Vpr and matrix allows its importation through nuclear pore into the cell nucleus. 7) The exposed hydroxyl group on cleaved 3′ ends cut through host DNA and viral DNA integrates into host chromatin. 8) When infected cell is activated, the provirus acts as a template for transcription of viral RNAs. 9) Spliced and unspliced viral RNAs are exported out of nucleus to cytoplasm for translation. 10) Viral proteins and genomic RNA assemble on plasma membrane. 11) The immature virion buds off cell membrane. 12) HIV-1 virion undergoes maturation under HIV-1 protease accompanied by structural changes of virion proteins to form a mature infectious HIV-1 virion.

1.3.1 HIV-1 binding and fusion

The first stage of HIV-1 infection is binding or attachment coordinated by surface envelope glycoprotein. HIV-1 env is around 400 kDa in size and trimeric in structure (trimers of gp120/gp41 complexes). Through coordinated events, the viral envelope glycoprotein attaches to its primary cellular $CD4^+$ receptor and subsequently to CXCR4 or CCR5 chemokine co-receptors. HIV-1 receptor $CD4^+$ marker is a member of the immunoglobulin superfamily that is expressed on cells including helper and regulatory T cells, CD4 T cells, macrophages, monocytes, and dendritic cells (177). The Van der waal forces and hydrogen bonds stabilise the complex of gp120-CD4 through interaction between the negatively charged gp120 pocket and positively charged CD4 receptor. The amino acid Phe 43 on CD4 receptor has been implicated in increasing the binding affinity of gp120 to CD4 receptor by 23%. The binding of gp120 to CD4 receptor triggers a change in conformation of gp120 which enables interaction with coreceptors, CCR5 (178) or CXCR4 (179). HIV-1 tropism is defined by the type of coreceptor usage during infection; CCR5 binding viruses (M-tropic) (178) CXCR4 (T-tropic) (179) and CCR5-CXCR4 (dual-tropic) (180). It has been shown that CCR5 mainly expressed on surface of macrophages is preferred during early infection and CXCR4 is largely preferred during late phase of HIV-1 infection. On binding of gp120 to its coreceptor CCR5 or CXCR4, triggers conformational change of transmembrane protein gp41. gp41 consists of fusion protein and N-terminal heptad repeat (HR) 1 and C-terminal HR2 regions. The fusion protein has hydrophobic glycine-rich residues which facilitate penetration of HIV-1 virion into target cell membrane (181). After binding of gp120 to its coreceptor, fusion protein is inserted into target cell membrane which triggers interaction between HR1 and HR2 regions to

form 6-helix structure which leads to formation of a fusion pore through which HIV-1 capsid enters into cytoplasm of $CD4^+$ cell (181).

1.3.2 Uncoating

After successful viral attachment and fusion, the cone-shaped viral capsid (CA) dissembles in the cytoplasm during the process of uncoating (182). The CA encompasses genomic RNA, viral and cellular proteins (50) and around 1500 CA molecules that oligomerize through interactions between their N-and C-terminals. Despite the timing of uncoating being not properly explained, the limited presence of CA molecules in the reverse transcription complex indicates early occurrence of uncoating (182). In support of this view, defects in viral core leads to impaired steps like reverse transcription (183–185). The viral core stability is critical for the uncoating as it affects the ability of HIV-1 to infect non-dividing cells (186) and nuclear import of PIC to the nucleus (187). The IN is among viral proteins facilitating CA uncoating (188). Host factors are also involved in uncoating of the viral core; TRIM5α impairs the process through increased output of uncoating (189,190) and recognition of CA lattice by TRIM5α activates innate immune signaling pathways (191). Cyclophilin A promotes uncoating of viral core (192,193) and interaction between cyclophilin A and CA leads to specific increase in infectivity in some cell lines (194–196). Host factors are also known to facilitate the shedding of the CA protein to release the two single-stranded viral RNA copies and accessory proteins inside the cell cytoplasm. Inside the cytoplasm, the viral single-stranded RNA must undergo reverse transcription to complementary DNA (cDNA) to facilitate viral replication. The viral reverse transcriptase enzyme and cellular factors are required for the process of reverse transcription to occur.

1.3.3 Reverse transcription

After viral entry into the host cell cytoplasm, the HIV-1 core slowly disassociates to reduce spatial constraints and allow for infusion of dNTPs into a PIC in which reverse transcription of the genomic RNA occurs. The host $tRNA^{Lys,3}$ primer already packaged and bound to genomic viral RNA (vRNA) anneals at its 18 nucleotide 3′ end to the primer binding site (PBS) found ~180

nucleotide from 5′ end of vRNA. The stability of vRNA and $tRNA^{Lys3}$ complex is mediated by complex, extended base pairing interactions that are likely facilitated and then stabilized by the nucleocapsid protein (197,198). The minus-strand cDNA is initiated from $tRNA^{Lys3}$ and becomes the first product of reverse transcription (minus strand strong stop DNA). RNaseH degrades the RNA portion of the newly formed RNA/DNA hybrid and releases the minus strand strong stop DNA. The complementarity repeat (R) of sequences at 5′ end of minus-strand strong stop DNA and 3′ of vRNA allows minus strand DNA to hybridize 3′ end of one of two vRNAs in HIV-1 particle and continue the synthesis of full-length minus-strand DNA, a process known as first template switch. During the minus strand DNA synthesis, the RNAse H activity of RT degrades the genomic RNA in RNA-DNA hybrid but retains a RNase H-resistant, highly conserved purine rich-polypurine tract (PPT) at the 3′ of genomic RNA and PPT acts as a primer for synthesis of the plus-strand DNA synthesis. The synthesis of positive-strand DNA is initiated at PPT and proceeds to the end of HIV-1 genome and it also copies the first 18 nucleotides of $tRNA^{Lys3}$ primer which becomes a substrate for RNaseH once it has been copied to DNA. HIV-1 cleaves tRNA from 3′ end leaving a single A ribonucleotide at 5′ end of minus-strand. The 18 nucleotides copied from $tRNA^{Lys3}$ are complementary to the 18 nucleotides at the 3′ end of minus-strand DNA copied from the pbs. The two complementary sequences anneal which allows synthesis of both positive and minus-strands to ends of templates completing proviral double-stranded DNA. This process is summarized in Figure 7.

The synthesized proviral vDNA becomes the specific substrate for the PIC. The PIC consists of viral proteins, RT, IN, matrix and Vpr (199–201) and ever increasing number of host proteins including, BAF, EED, HMGA1, p300, and LEDGF/p75, and HRP-2 (202).

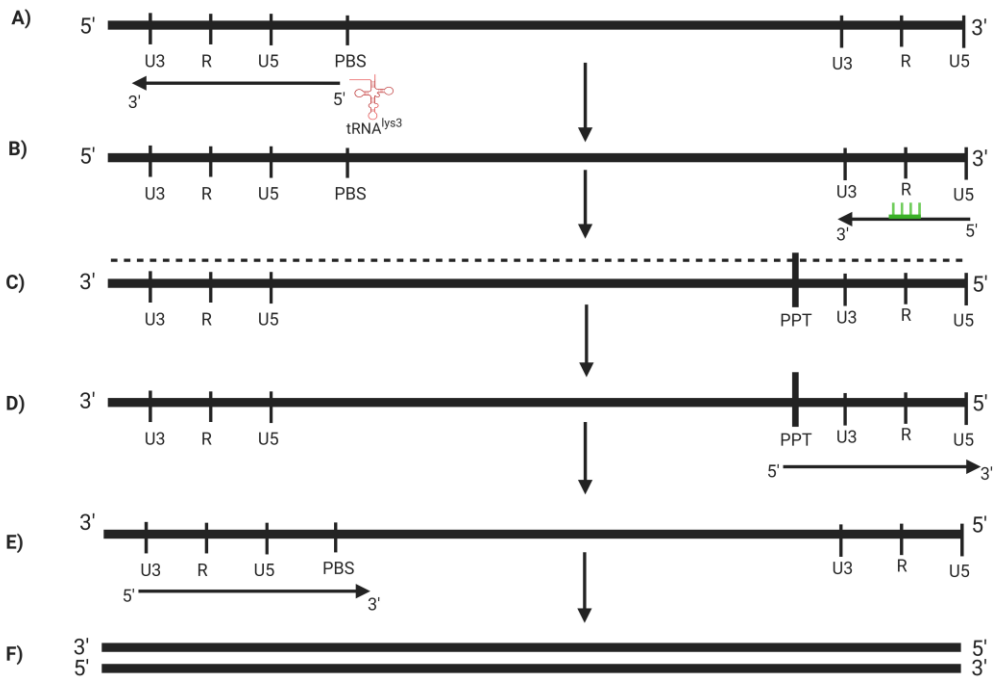

Figure 7. HIV-1 reverse transcription. HIV-1 is a retrovirus with two copies of viral RNA which are reverse transcribed into double-stranded DNA for integration into human genome. A) In the first step of reverse transcription, human $tRNA^{lys3}$ binds to the complementary sequence on pbs region of 5′ of viral RNA. The binding of $tRNA^{lys3}$ at pbs initiates synthesis of minus -strand DNA. B) The complementarity repeat (R) of sequences at 5′ end of minus-strand strong stop DNA and 3′ of vRNA allows minus strand DNA to hybridize 3′ end of one of two vRNAs. C) The binding at the R allows synthesis of full-length minus-strand DNA. In the process, the original viral RNA template is degraded by RNaseH except for the RNaseH-resistant highly conserved purine rich-polypurine tract (PPT) at the 3′ of genomic RNA which subsequently acts as a primer for synthesis of the plus-strand DNA synthesis. D) The synthesis of positive-strand by RT is initiated at PPT and proceeds to the 5′ end of viral genome including 18 nucleotide sequence of $tRNA^{lys3}$. E) The complementarity of $tRNA^{lys3}$ sequence allows annealing of positive strand on pbs. F) The annealing of two complementary sequences allows synthesis of both positive and minus-strands to generate proviral double-stranded DNA.

1.3.4 3′ processing of terminal vDNA sequences

The HIV-1 IN recognizes and binds to short specific sequences (12-20 bp) on both 3′ ends of viral LTRs. This high-order nucleoprotein complex of linear vDNA and HIV-1 IN known as intasome mediates integration of HIV-1 into target DNA. Reverse transcription of genomic RNA by reverse transcriptase results a blunt-ended vDNA duplex with terminal sequences 5′-ACTG**CAGT**-3′. The ‘TGCA’ repeat is conserved in all retroviruses, retrotransposons, and in some prokaryotic DNA

transposable elements. During 3′ processing which occurs in the cytoplasm (203), and in PIC (204), HIV-1 IN catalyzes the cleavage of dinucleotide (GT) from both 3′ ends of vDNA at conserved CA and exposed CA_{OH}-3′ acts as nucleophiles for cleavage of target DNA later during strand transfer process in the nucleus (51,52) (Fig. 8).

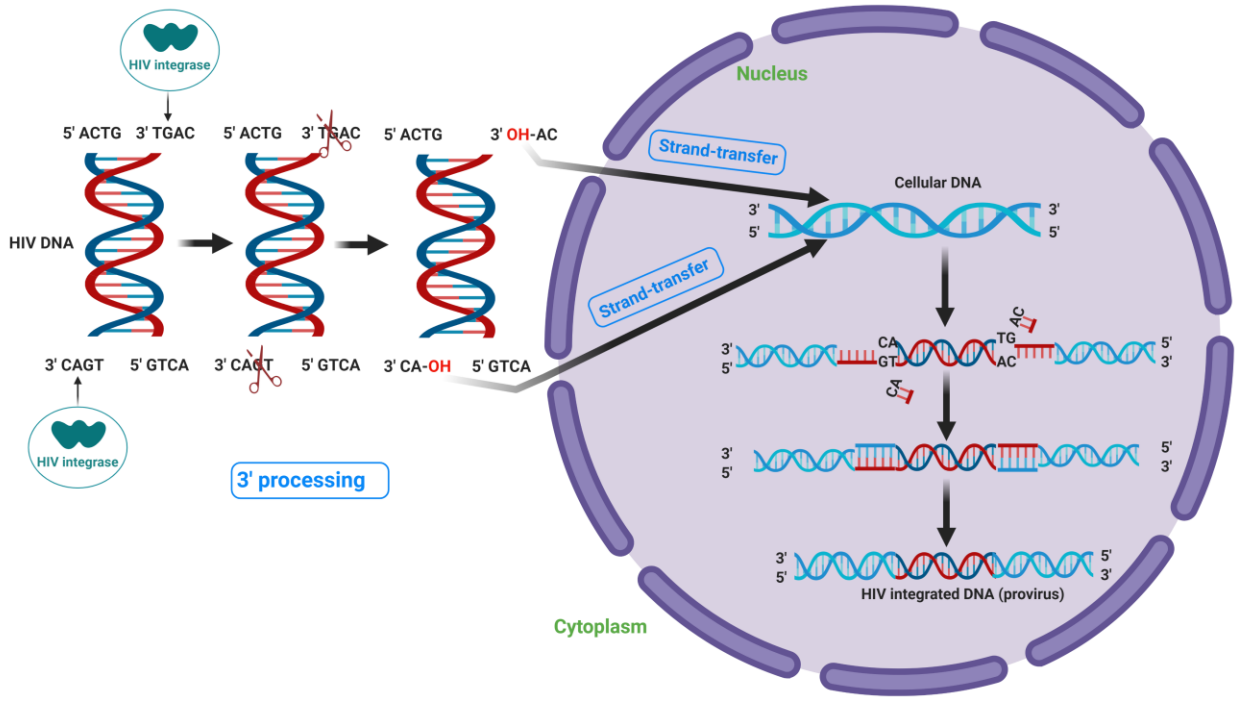

Figure 8. HIV-1 3′ processing and strand transfer processes. HIV-1 integrates into the host chromatin in a two-step process involving cleavage of dinucleotide 'GT' at conserved CA site of 3′ LTR of HIV-1. The HIV-1 integrase remains bound to cleaved viral DNA which is translocated into the nucleus in pre integration complex. During strand transfer, the exposed hydroxyl act as nucleophiles and cut through phosphodiester bond of the phosphate at 5′ end of host DNA in staggered fashion creating 5-base pair insertion. The lone CA is removed and cut DNA ligated by cellular proteins which completes integration process.

1.3.5 Import of PIC into nucleus

The HIV-1 PIC is formed after viral core uncoating and formation of cDNA from reverse transcription of viral RNA. The HIV-1 PIC has karyophilic properties which it inherits from three of its components, HIV-1 Gag matrix (205,206), Vpr (207), and IN (48). In addition, PIC

associated cellular proteins, barrier-to-autointegration factor (BAF), HRP-2, and LEDGF/p75 contain the nuclear localization signals (NLS) which allow active transport of PIC via nuclear pore (less than 40 kDa) into the cell nucleus. The karyophilic property of the HIV-1 PIC accounts in part for the ability of HIV-1 to infect both terminally differentiated macrophages and G2-and S-phase arrested cells (206,208). The MA has a stretch of highly conserved amino acids, Gly-Lys-Lys-Lys-Tyr-Lys (209), which is recognized by importin/karyopherin proteins (205,210). Indeed, a synthetic peptide containing putative NLSs of MA acted as nuclear import signal when combined with bovine serum albumin contrary to abolished nuclear import when the peptide was conjugated to wheat-germ agglutinin, an inhibitor of nuclear pore-mediated import heterologous protein *in-vitro* (205). The phosphorylation of carboxyl-terminal tyrosine residue of matrix protein may be required for direct nuclear export of PIC (211). Nuclear import of PIC is further facilitated by several importins which transport proteins with NLS through nuclear pore. Importins, Imp7 (212), Impα1 (48,213), Impα3 (49,214), and Transportin 3(TNP03) directly bind to HIV-1 IN in cells in interphase to promote nuclear import of PIC (215,216). Nucleoporins, Nup153 interacts and facilitate import of HIV-1 Vpr (217) and HIV-1 IN (218), whereas Nup358 specifically binds to HIV-1 Rev and enables cDNA nuclear import (219). The role of HIV-1 Vpr for nuclear import of PIC may be stimulated by heat shock protein 70 (Hsp70) (220). In dividing cells, the HIV-1 PIC targets disassociation of cell nuclear membrane during mitosis to enter nucleus during interphase stage by signal mediated and energy dependent import through nuclear pore (208,221,222).

1.3.6 Strand transfer-integration

Once inside the nucleus, the PIC is tethered to host DNA by several cellular proteins including LEDGF/p75, BAF, Integrase interactor 1 (INI-1), and high mobility group protein A1 (HMGA1). The most well characterized of these is LEDGF/p75 (223–226). LEDGF/p75 is a 530 amino acid protein that acts as a transcriptional factor and a survival factor encouraging cell growth by preventing stress-induced cell death. LEDGF/p75 is localized to the nucleus where it is found in tight association with chromosomes (227), and its nuclear abundance perfectly matches that of HIV-1 IN (228). The close contact of LEDGF/p75 with chromosomes helps to tether PIC to highly transcribing regions of cellular DNA (229,230). The role of LEDGF/p75 in HIV-1 integration is further demonstrated by its up-regulation in HIV-1-infected cells (231) and increased strand-

transfer activity exhibited by recombinant HIV-1 IN in the presence of recombinant LEDGF/p75 *in vitro* (228). In addition to tethering PIC to chromatin, LEDGF/p75 increases the HIV-1 IN half-life by preventing its ubiquitination and degradation by proteasomes (232).

BAF is an 89 amino acid protein that bridges DNA molecules in a nucleoprotein complex by binding double stranded DNA. BAF is recruited in PIC (233) and has been implicated in orientating the PIC to chromatin (234–237). BAF also prevents autointegration of cDNA before integration (238). Lamina-associated polypeptide 2 alpha (LAP2alpha) is also involved in positioning PIC to chromatin by interacting with BAF (236). PIC associated HMGA1 protein has been implicated in facilitating DNA unwinding, supercoiling and bending. HMGA1 shows no interaction with HIV-1 IN but may facilitate HIV-1 integration by bringing vDNA ends together and into close contact with HIV-1 IN. INI-1 is a 385 amino acid protein that interacts with HIV-1 IN and activates HIV-1 LTR by synergizing with tat and p300/CBP at 5′ HIV-1 LTR.

In the nucleus and following dinucleotide cleavage/processing of the 3′ of vDNA, integratable vDNA associated with the PIC will mediate proviral DNA integration into the host genome. The IN bound to exposed 3′-OH ends of vDNA now cuts the host DNA by breaking down a pair of the phosphodiester bonds via nucleophilic attack by the reactive 3′-OH of vDNA (Fig.7). The host DNA cuts occurs simultaneously at both strands in a staggered fashion typically at position separated by 5 base pairs between the two opposite ends of insertion, exposing the 5′ phosphate end of host DNA that is ligated to the 3′-OH of the vRNA (239). After strand transfer, the vDNA and host DNA junctions have a 5 base pair sequence gap on the leading and lagging DNA strand in addition to 2 base pairs on 5′ of vDNA that remain unpaired. In the subsequent step of HIV-1 integration, the host cellular enzymes remove 5′ dinucleotide (CA) ‘flaps’ on vDNA and catalyze the filling of the gaps to complete integration process (240,241). The PIC is dissembled within minutes following joining of vDNA 3′ ends to the target DNA which results in an irreversible process of proviral integration. The cleavage of host DNA mediated by HIV-1 IN and ligation of vDNA and host DNA doesn’t require exogenous energy source. In addition to 3′ processing and strand transfer activities, HIV-1 IN can cleave integrated vDNA from host DNA *in vitro* in process termed disintegration and this can be accomplished in absence of either NTD or CTD (242–245). As described above and possibly related to LEDGF/p75 binding, HIV-1 proviral DNA is preferentially integrated into host DNA in regions of high transcription activity, which is believed

to facilitate virus infection and transmission (231). The provirus serves as a template for transcription into mRNAs in a process catalyzed by host RNA polymerase II or goes into transcriptionally inactive phase called latency. The latency phase is characterized by transcriptionally silent cells which don't produce virions (246).

Not all proviral DNA circularizes and associates with PIC/IN as a substrate for proviral DNA integration. Unintegrated extrachromosomal vDNA can also be found as linear (3′ processed cDNA) and circularized DNA unintegrated forms inside the cell nucleus (Fig. 9). The circularized DNAs have covalently closed DNA circles with either single LTR sequence or two LTR sequences commonly referred to as 1-LTR circles and 2-LTR circles respectively. The 1-LTR circles are formed earlier than 2-LTR circles (247), and occur in most abundance compared to other forms of unintegrated DNA with the ratio of 2-LTRs to 1-LTRs circles ranging from 0.16-0.43 (248). The 1-LTRs circles are products of homologous recombination between two flanked HIV-1 LTRs (248) or circularization due to defective reverse transcription (249). About 90% of 1-LTR circles are products of homologous recombination which occurs after nuclear import and are commonly found inside the nucleus (248). Approximately 10% of the total 1-LTR circles population is found in the cytoplasm, and these are products of defective reverse transcription (247). Cellular nucleases, RAD50, MRE11, and NBS1 have also been implicated in formation of 1-LTR circles (248,250). Another fate of unintegrated linear viral DNA, is autointegration which happens when HIV-1 IN mediates 3′ processing of cDNA LTR ends which attack sites within viral DNA (self-integration), resulting in circularized forms of rearranged or incomplete vDNA genomes (251) (Fig. 9).

The 2-LTR circles are products of non-homologous end joining (NHEJ) pathway (252) and autointegration process, and they are exclusively formed inside the nucleus (247). The host NHEJ components are involved in double-strand DNA break repair during the $G_{0,}$ G_1 and early phase of the cell cycle as a protective strategy towards presence of viral ssDNA. The NHEJ pathway involves binding and holding of ends of broken DNA by DNA-dependent serine/threonine protein kinase (DNA-PK) consisting of autoimmune antigen ku and DNA-PK catalytic subunit (DNA-PKs). With ku guiding DNA-PKs to cut DNA ends, ligase 4/XRCC4/XLF performs the ligation reaction (253,254). In absence of compatible DNA ends for ligation, Artemis nuclease often trims the ends to facilitate ligation by polymerases (255). The role of NHEJ components to the formation

of 2-LTRs is shown by decreased abundance of 2-LTRs when Ku, ligase 4 or XRCC4 are inactivated and modest reduction is observed upon inhibition of DNA-PKcs (248,256). Two LTR circularized DNA forms can still mediate HIV-1 mRNA transcription (257), production of Tat and Nef proteins (258), and are found at higher frequency in macrophages and resting $CD4^{+}$T cells (259).

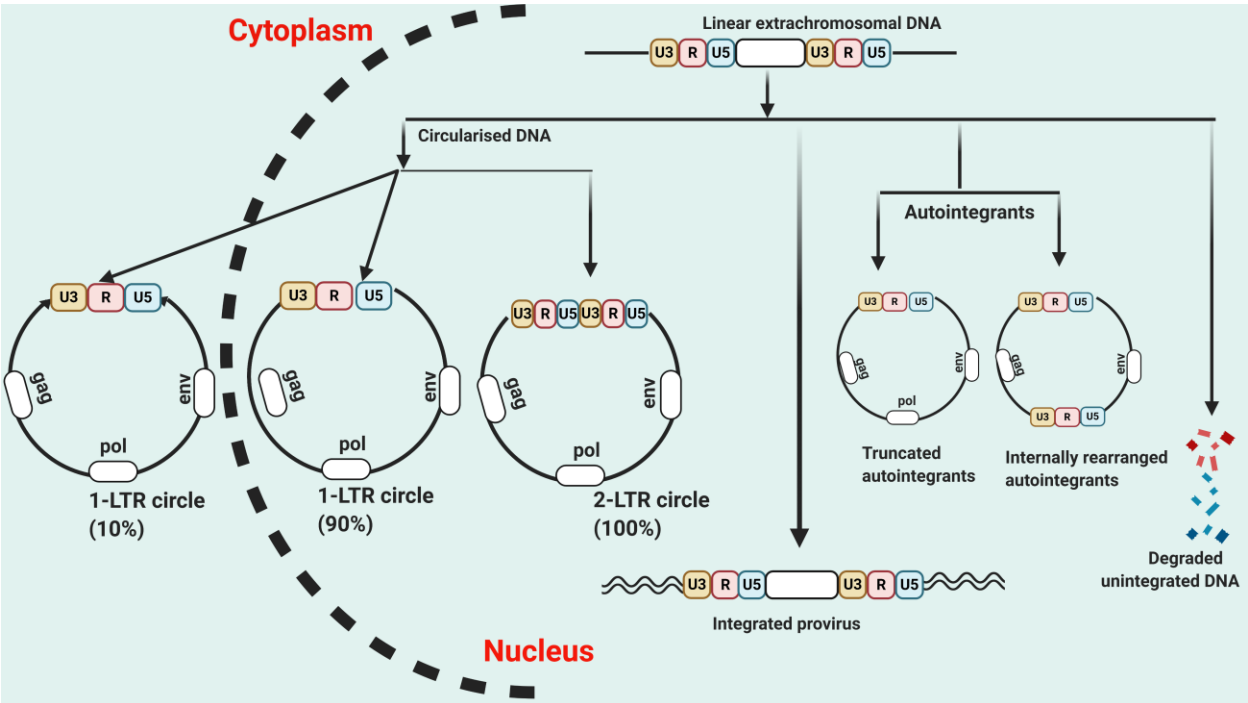

Figure 9. Unintegrated forms of HIV-1 DNA. The translocated cleaved viral DNA has two fates in the nucleus; successful integration into the host DNA to form a provirus or failed integration which is characterized by mainly two forms of circulating DNA-1 LTR circle or 2-LTR circle. The larger portion of 1-LTR circles (90%) is found in the nucleus and the rest is in the cytoplasm. 2-LTR circles are exclusively found in the nucleus. Another fate of unintegrated linear cDNA is autointegration due processed 3′ ends of reverse transcripts cleaving sites within viral DNA resulting into truncated and internally rearranged autointegrants. The majority of unintegrated viral DNA is degraded by various host DNA repair and restriction factors.

1.3.7 Transcription, nuclear export of nascent RNA, translation and virus assembly

Transcription of provirus is regulated by the promoter region of HIV-1 5′ LTR which contains crucial DNA binding sites; two NFkB sites, three Sp1 sites, and a TATA box. Upon stimulation of CD4+ T cell, NFAT and NFkB are activated and recruited to the promoter region of nascent RNA transcripts. This leads to recruitment of Tat to the promoter region which trans-activates HIV-1 mRNAs expression by interacting with Trans acting response element (TAR) of 5′ HIV-1 LTR. Tat binding on TAR stabilizes TFIID/TFIIA complex on the TATA box and facilitates recruitment of TATA-binding protein, and a cellular P-TEFb and CTD kinases to the HIV-1 promoter. These kinases include CDK family of CDK2, CDK7, CDK8, CDK9, and CDK11. P-TEFb is a heterodimer protein made up of cyclin-dependent kinase 9 (CDK9) and its regulatory partner cyclin T1. The human cyclin T1 mediates interaction between P-TEFb and Tat (260). In presence of Tat, CDK9 phosphorylates the CTD domain of RNA pol II which increases processivity of elongating RNA polymerase II (261). In the absence of Tat, only serine 2 of heptapeptide repeats is phosphorylated by CDK9 and serine 5 by a component of TFIIH, CDK7 (261). Tat also interacts with transcriptional cofactors including sp1, cyclinE/cdk2, TFIIH, Tip60, polymerase II and transcription activators p/CAF and CBP/p300. In the absence of Tat, the rate of HIV-1 transcription initiation is not affected but no elongation of mRNA transcripts is observed beyond +59 position due to pausing by RNA pol II (176).

Alternative splicing of HIV-1 mRNA leads to 47 different RNA species. Depending on extent of splicing, there are three categories of HIV-1 RNA transcribed from provirus: 1) partially spliced RNA, which are ≈ 4 kb length transcripts of Vpu, Env, one exon Tat, Vpr and Vif; 2) completely spliced, ≈ 2 kb transcripts of Tat, Rev, Nef; 3) unspliced RNA, genomic RNA for packaging, Gag, and Gag-Pol polyproteins. In the early phase of nuclear export, 9 kb mRNA transcripts are spliced to 2 kb RNAs which are exported via normal mRNA pathway and are translated to produce Tat, Rev, and Nef. After translation, Rev is imported back to the nucleus *via* its nuclear localization signal(169,262). In the late phase of nuclear export, Rev binds partially spliced and unspliced mRNAs at Rev Response element (RRE) located in *env* coding region before splicing, and exports these unspliced or partially spliced messages out of the nucleus to cytoplasm. The leucine rich nuclear export signal of Rev enables recruitment of Crm1 protein to mediate this

nucleocytoplasmic export. To facilitate nuclear export of cargo, Rev also recruits nucleoporins, Nup98 and Nup124 in rev-Crm1 controlled export (263). The partially spliced and completely spliced RNAs are shuttled to the ribosome in the cytoplasm for translation into various viral proteins.

The Env and Vpu proteins which are coded by the same mRNA transcript are translated on rough endoplasmic reticulum (RER). The Env proteins are glycosylated as they translocate through membrane of RER, and they are cleaved by Furin protease to produce SU gp120 and transmembrane protein gp41 in the Golgi apparatus, and then transported to the plasma membrane. At budding site (cell membrane), the intracellular tail of the transmembrane Env gp41 interacts with MA region of the Gag and Gag-Pol precursor proteins. Gag also binds, interacts with, and packages other components into a newly formed core. These include (264,265) cellular proteins, ICAM-1, HLA-II, cyclophilin A, actin-binding proteins, lysyl tRNAs-synthetase, and ubiquitin (266). HIV-1 Gag preferentially binds and incorporates/encapsidates two copies of the genomic HIV-1 RNA while the Gag-Pol is involved in the preferential packaging and placement of the $tRNA^{Lys3}$ on the genomic RNA. During assembly, Env protein, PR, RT and IN viral enzymes are incorporated into the immature virion. The HIV-1 particle later buds off of the cell surface as an immature virion mediated by the host endosomal sorting complexes required for transport (ESCRT) machinery.

1.3.8 Maturation of viral proteins

Maturation of HIV-1 particle occurs simultaneously with or immediately after budding from the cell surface and is triggered by proteolytic cleavage of Gag and Gag-Pol precursor proteins by HIV-1 PR. During virion maturation, the PR subunit in the Gag-Pol precursor autocleaves itself out and proceeds to cleave the rest of Gag-Pol and Gag polyproteins at twelve different sites into mature structural MA, CA, NC, P6, small spacer peptides p1 and p2, and catalytic enzymes PR, RT, and IN (45). This cleavage at these different sites occurs sequentially and at different rates with e.g. the first cleavage occurring between spacer peptide p2 and NC and intermediate cleavage occurring between CA/MA and p1/p6 sites, and final cleavage at the NC/p1 and CA/p2 sites (16,27,267–270). The fully processed structural and catalytic enzymes get dramatically rearranged

within the viral particles to form the mature viral particles. Following its release from the Gag-Pol polyprotein, CA forms hexameric lattice that forms the conical viral core containing viral RNA genome, mature NC, RT, and IN proteins (Fig 1). The sequential maturation process of Gag and Gag-Pol is described in Figure 3.

1.4 Antiretroviral therapy

The first report of a new deadly disease found in men who have sex with men in 1981 and then among hemophiliacs and intravenous drug users in the USA (2,271,272) led to the rapid search for a drug to block this unknown disease. The discovery that AIDS was caused by HIV-1 in 1983 then led to the rapid testing of nucleoside analogs that had been shown to inhibit avian and murine retroviruses. Among the nucleoside analog class, only acyclovir, an acyclic nucleoside analogue against herpes simplex was approved for therapy by the FDA and was the only effective antiviral agent available on market (273,274).

3'-azido-3'-deoxythymidine (AZT), first synthesized in 1964, was the leading agent among nucleoside reverse transcriptase inhibitors (NRTIs) (275,276) and was rapidly approved in 1987 (Fig. 10) for HIV-1 treatment after undergoing a small exploratory study (277) and a double blind-placebo controlled trial. Drug resistance quickly emerged in patients taking AZT (278). In subsequent years, more NRTIs were available for HIV-1 treatment including didanosine (2',3'-dideoxyinosine) (279) and zalcitabine (2',3'-dideoxycytidine) (280) which allowed comparison of dual NRTIs to monotherapy. Better outcomes in antiretroviral therapy (ART) naïve patients were recorded with dual nucleoside analog therapy (281–284) which paved a way for triple therapy-highly active antiretroviral therapy (HAART) in 1996 after a number of studies including ACTG 320 which showed adding two new drugs (indinavir (IDV) and lamivudine (3TC) with AZT in AZT experienced patients was more effective than adding a single drug (285). HAART combinations differ in each geographical location and largely attributed to the disproportional ability to access the most potent antiretroviral drugs for the most effective treatment regimens by countries of different economic means. This inequality for HIV-1 treatments across the globe remains today after 25 year of effective HAART, now referred to as combined ART (cART).

Currently, there are over 34 individual and 23 HIV-1 drug combinations approved by Food and Drug Authority (FDA) for therapeutic treatment and one ART combination for HIV-1 prevention (Fig. 10). Over the years, cART have become more potent, formulated in a single daily pill, are easier to tolerate with few adverse events, and high barrier to drug resistance, all of which has improved treatment outcomes. These new formulations include fixed dose combinations which contain two or more drugs from two or more drug classes in a single tablet. Long acting injectables which are dosed once per week or month are tolerable and improve adherence to ART (US national library of medicine). These may include long acting injectable, cabotegravir/rilpivirine (in late phase III clinical trial), dapivirine within a vaginal ring, and tenofovir alafenamide as a subdermal implant (286–288) (289). ART regimens reduce viral replication by targeting different stages of HIV-1 life cycle.

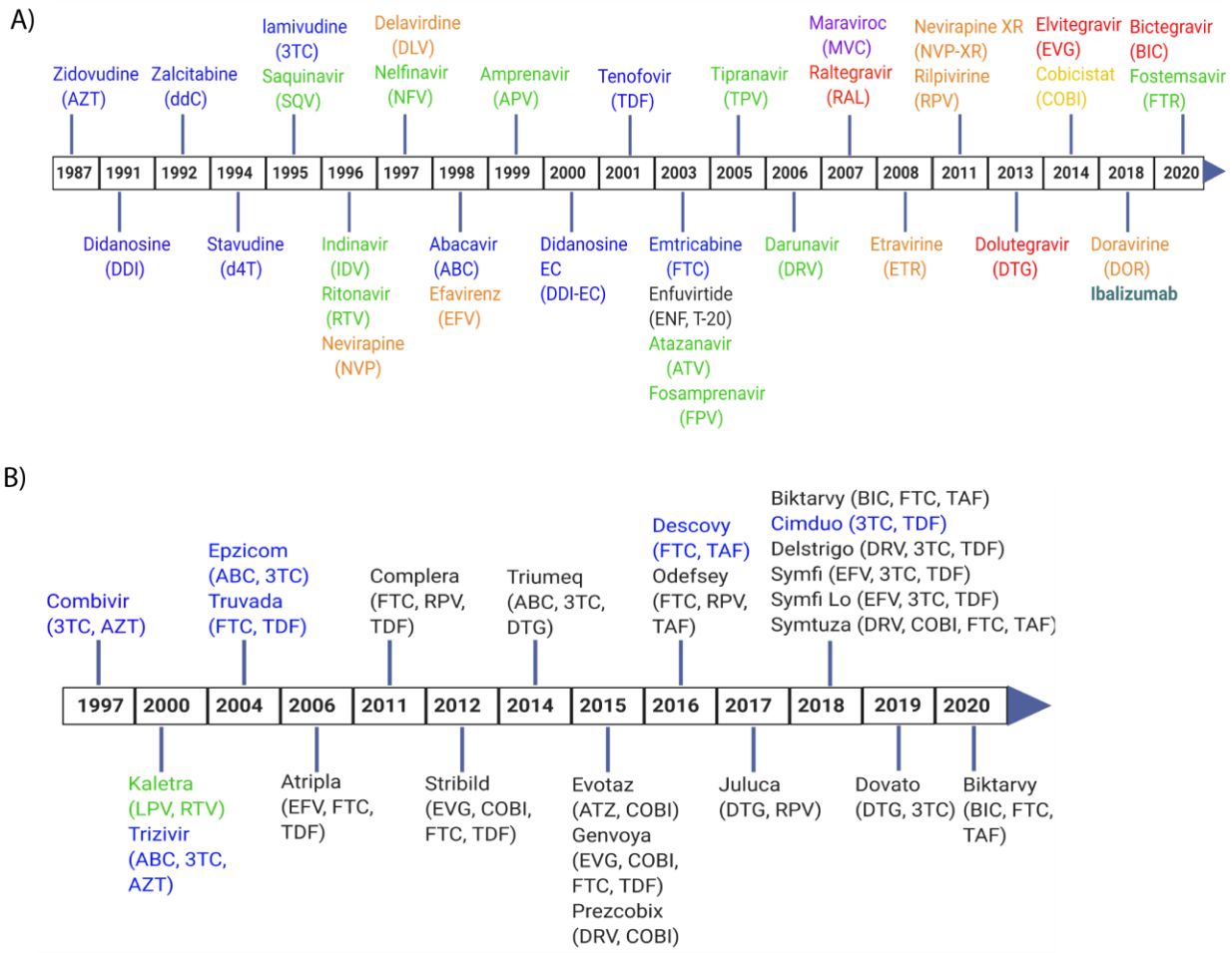

Figure 10. FDA approved HIV-1 treatment drugs and combinations. A) Regimens approved for HIV-1 treatment, in blue, nucleoside reverse transcriptase inhibitors; orange, non-nucleoside reverse transcriptase inhibitors; green, protease inhibitors; red, integrase inhibitors; yellow, pharmacokinetic enhancer; brown, post attachment inhibitors; and black, fusion inhibitors. B) Brand names of fixed-dose tablets with drug abbreviations. In blue, combinations consisting of reverse transcriptase inhibitors; green, combinations of protease inhibitors; black, combinations with more than one class of ART.

There are mainly 5 classes of ART; reverse transcriptase inhibitors target reverse transcription step, protease inhibitors target cleavage of polyprotein gag-pol and gag by protease, integrase inhibitors target strand transfer step, fusion inhibitors target fusion of gp41 onto host cell membrane, and binding inhibitors target HIV-1 coreceptor CCR5. Along with a novel long acting anti-CD4 monoclonal antibody, Ibalizumab, new agents targeting capsid assembly, glycoprotein 120 attachment, Rev-dependent mRNA expression, present alternative pathways to viral replication inhibition.

1.4.1 Classes of ART and mode of action

1.4.1.1 HIV-1 integrase strand transfer inhibitors

HIV-1 integrase strand transfer inhibitors (INSTIs) target HIV-1 IN to stop viral replication. HIV-1 IN catalyses HIV-1 integration by 3′ endonucleolytic processing of vDNA ends in the cytoplasm and strand transfer process in the cell nucleus where vDNA is covalently linked to cellular DNA. The discovery of integrase inhibitors depended on screening compounds using assays where recombinant HIV-1 IN catalyzed the first step of dinucleotide cleavage on the end of viral DNA and/or the strand transfer reaction. The safety of HIV-1 IN is largely based on the fact that the host does not produce an enzyme with this type of endonucleolytic processing and strand transfer event. After this enzymatic assay screen, an effective IN inhibitor had to meet four basic preclinical development criteria in cell culture: (1) efficacy *in vitro* that was comparable to blocking HIV-1 replication in tissue culture (also suggesting cell penetration by the drug) (290–293), (2) accumulation of 2-LTRs and reduced integration into host chromosome (290,292,294), (3) with dose escalation experiments, the emergence of drug resistant viruses with mutations in integrase (290,293,294), and confirmation that the presence of DRMs in recombinant IN showed reduced susceptibility to the inhibitor *in vitro* (290,294).

The two strategies considered in development of HIV-1 IN inhibitors were; binding to free unbound HIV-1 IN or to HIV-1 IN-vDNA complex. Both strategies were attempted, however, the high stability of HIV-1 IN-vDNA complex characterised by slow catalytic activity makes it difficult for HIV-1 IN inhibitors to bind free HIV-1 IN (295). The complex of HIV-1 IN CCD and shionogi inhibitor 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone-5CITEP, formed a platform for design of HIV-1 IN inhibitors. The 5CITEP inhibitor binds centrally between D-D-35-E motif and forms hydrogen bonds with residues Thr 66, Gln 148, Asn 155, Lys 156, and Lys 159 (296) (Fig. 11). Some of these residues Lys-156, Lys-159, and Gln-148 are involved in vDNA binding (297,298). 5CITEP does not chelate cationic ions at the active site but tends to mimic DNA substrate/IN interaction and therefore seems to prevent binding of the second divalent cation by occupying the DNA-substrate binding site that inhibits the formation of HIV-1 IN/DNA complex (296,299) (Fig.11). The assays to identify ligands that bind to HIV-1 IN-vDNA complex led to the discovery of first INSTIs, diketo acids L-731,988 and L-708,906 (290).

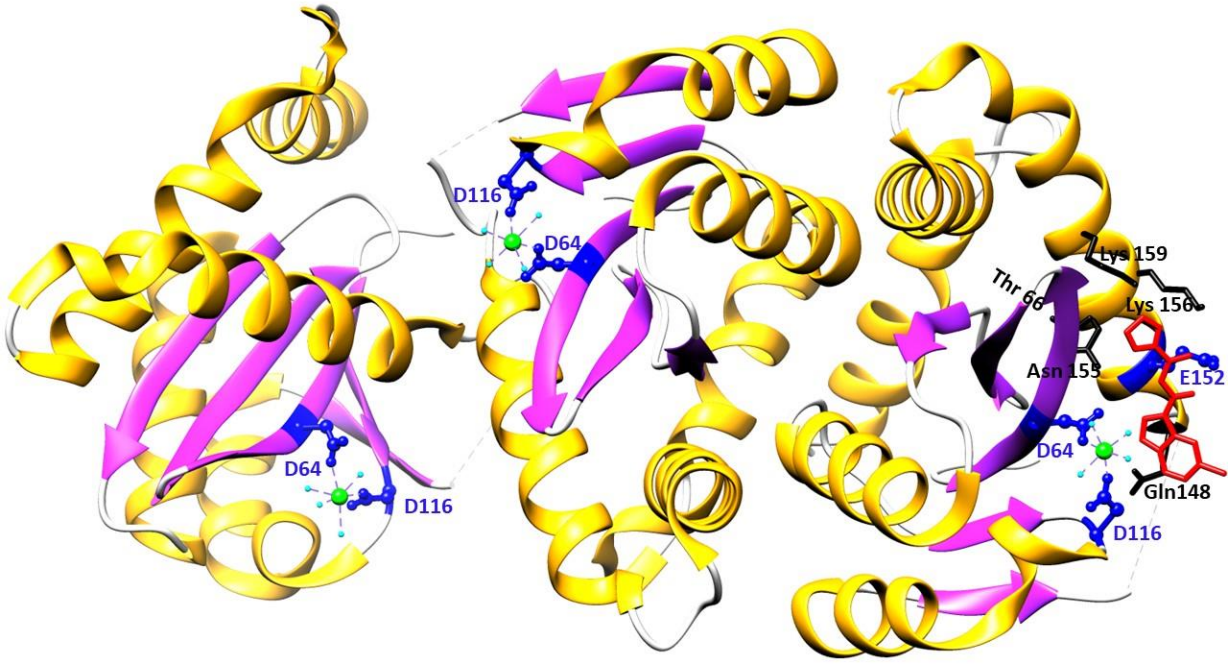

Figure 11. The HIV-1 IN CCD and 5CITEP complex. The crystal structure (PDB ID 1QS4) (296) shows three HIV-1 IN CCD monomers with HIV-1 IN inhibitor, 5CITEP (red) binding to one of the CCD monomers. The three catalytic residues (D64, D116 and E152) are depicted in blue and magnesium ion (green) being coordinated by D64, and D116 residues in presence of four water molecules (cyan). The HIV-IN residues which form hydrogen bond with 5CITEP inhibitor are shown in black.

Following this initial discovery and preclinical development of HIV-1 IN inhibitors at Merck, most INSTIs in development and all those approved for treatment target HIV-1 IN protein by specifically inhibiting strand transfer process not 3′ processing (300–304). HIV-1 IN requires a metallic cationic cofactor to be active and these are coordinated by a triad of acidic residues called DDE motif (D64, D116, and E152) in the catalytic domain of HIV-1 IN. INSTIs target the Mg^{2+} chelating groups due to the fact that Mg^{2+} is a cofactor required *in vivo* and is less tolerant to sequence variations in LTRs (305), and several mutations affect IN activity in Mg^{2+} dependent assays for instance mutations in zinc coordinating motif, HHCC of N-domain of HIV-1 IN (306). After successful cDNA synthesis by HIV-1 RT, IN recognises specific sequences at either end of

vDNA to form stable complexes with LTR DNAs (PIC). The confirmation change as a result of HIV-1 IN binding to vDNA allows the binding of INSTIs (307).

INSTIs bind to the CCD domain on IN, involved in chelated Mg^{2+}, and compete with host DNA substrate (307). They typically bind to IN/DNA complex close to 3′ end of vDNA in the enzyme and thus inhibits binding of donor DNA to the target host DNA (300,308) (Fig. 12).

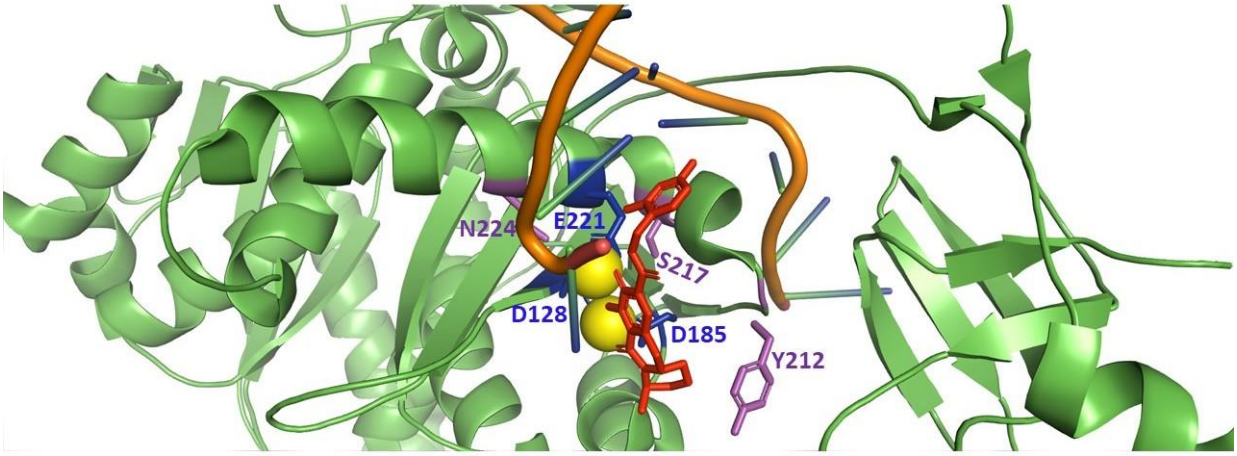

Figure 12. The crystal structure of DTG bound to prototype foamy virus integrase and vDNA. The structure shows DTG binding at active site of CCD of PFV IN with catalysis residues D128 (HIV-1, D64), D185 (HIV-1, D116) and E221 (HIV-1, E152) depicted in blue and divalent Mg^{2+} shown in yellow. DTG is shown in red binding to 3′ end of PFV vDNA. The common INSTIs associated resistance residues, Y212 (Y143, HIV-1), N224 (N155, HIV-1), and S217 (Q148/G140, HIV-1) are shown in magenta. The image was made in Pymol using x-ray crystallography structure (PDB ID 3S3M) (308).

For the INSTIs compounds to be active pharmacophores, they must have a chemical group of three oxygen atoms in same geometric plane and halogenated phenyl group in their chemical structure (Fig.13). The halobenzyl moiety fit within a tight catalytic pocket of HIV-1 IN made available after 3′ processing, and interact with G:C proceeding the terminal adenine of 3′ HIV-1 LTR viral end, and with residues 145 and 146 of HIV-1 IN. This interaction results in displacement of the reactive adenine (309,310) and eventually of 3′ hydroxy group from the active site which inactivates the intasome and stabilises the HIV-1 IN-vDNA complex. The stability of HIV-1 IN-

vDNA complex due to displaced 3′ hydroxy group is further elucidated by increased rate of association of INSTIs binding to HIV-1 IN-vDNA complex on deletion of this adenine base (311). The three oxygen atoms of β-hydroxy ketone moiety chelate divalent cations within the catalytic pocket which impedes catalytic triad D-D-35-E from participating in strand-transfer process (312–315).

Raltegravir (RAL), formerly known as MK-0518, was the first INSTI to be approved by the FDA in 2007 (316,317), followed by elvitegravir (EVG) (JTK-303/GS-9137) in 2012, dolutegravir (DTG) in 2013 (S/GSK1349572), bictegravir (BIC) (GS-9883) in 2018 and cabotegravir (CAB) formerly S/GSK 1265744 or GSK 744 in 2021. Both first generation INSTIs, RAL and EVG are effective against WT HIV-1 but the genetic barrier to drug resistance was less than that observed with the second generation of INSTIs (DTG, BIC and CAB). DTG and BIC remains effective in the presence of some HIV-1 resistant to RAL and EVG (318,319). The structural differences between DTG and RAL or EVG may explain high potency seen with DTG. Contrary to EVG/RAL, the chelating motif of DTG is located on a tri-cyclic scaffold (319,320). The linker region joining metal chelating motif to halogenated group of DTG is further extended compared to RAL and EVG (308) (Fig.13) which allows deeper access into the pocket vacated by displaced vDNA allowing a closer interaction with vDNA. In addition, presence of RAL/EVG resistant viruses, DTG can adjust its position and conformation in response to structural changes at the IN active site.

INSTIs, BIC, and CAB, are the latest second generation INSTIs. BIC a structural analog of DTG was approved in 2018 as a fixed-dose combination of BIC/FTC/ tenofovir alafenamide (TAF) in ART naïve and suppressed patients (<50 copies/ml) (321). BIC is a potent, un-boosted, once daily INSTI, with higher *in vitro* barrier to resistance against RAL and EVG and with a few drug-drug interactions. CAB is another analog of DTG from a class of carbamoyl pyridones that has recently been approved by FDA and European Union as long-acting injectable, CAB/rilpivirine for use in HIV patients with undetectable viral loads, stable on current ART, and no drug resistance. [9–11] The unique physicochemical and pharmacokinetic properties of the CAB formulation allows its use as a single-daily tablet or long-acting nanosuspension for monthly or quarterly via subcutaneous or intramuscular administration. CAB was recently approved as a fixed-dose long-acting injectable combination of CAB plus the NNRTI rilpivirine after successful clinical trials in HIV infected

individuals. [12,13]. BIC and CAB are structurally and chemically similar to DTG both having tri-cyclic central pharmacophore, though metal-chelating scaffold for CAB is 5-membered (more rigid) compared to 6-membered ring (more flexible) for DTG (Fig.13) (325). Overall, BIC seems to be more effective than either CAB or DTG which may be explained by further flexibility of oxazepine ring allowing accommodation to structural changes of IN active site in presence of resistant mutations.

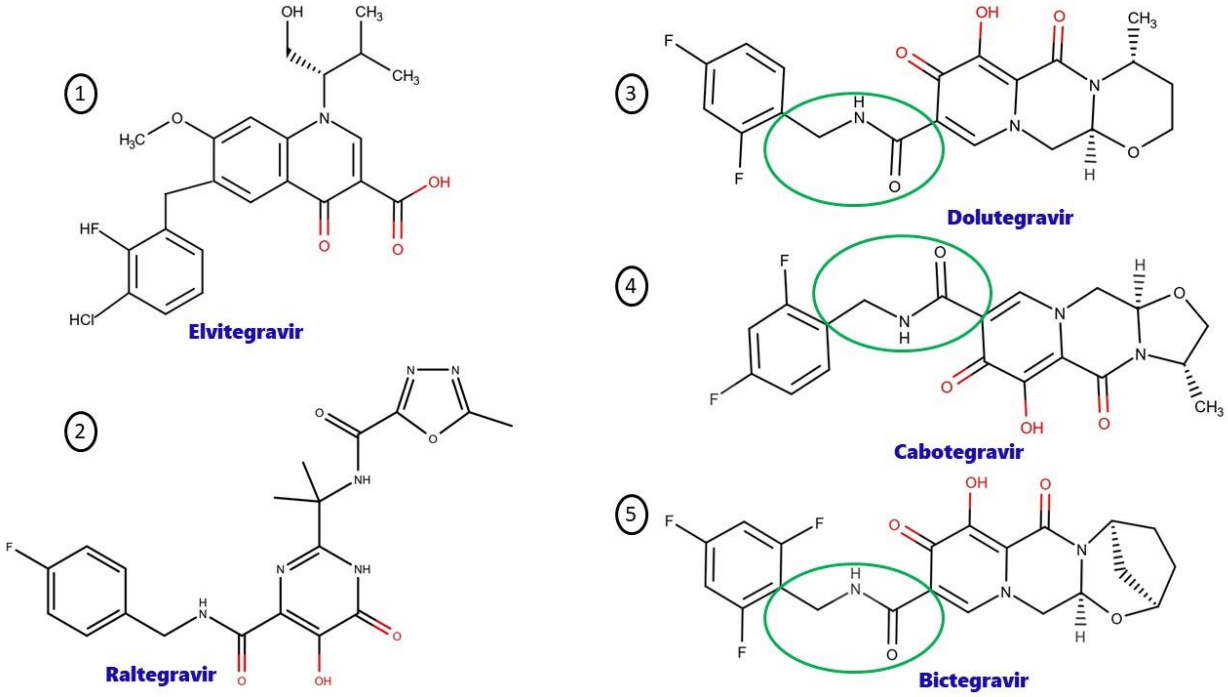

Figure 13. HIV-1 integrase strand transfer inhibitors (INSTIs). Currently, there are two groups of INSTIs, the first generation (RAL and EVG) and second generation INSTIs (DTG, BIC, CAB). All currently approved INSTIs are characterized by three coplanar oxygen group for chelating magnesium ions at active site of HIV-1 IN and halogenated benzyl group which seats in pocket left by cleaved 'GT' dinucleotide and eventually displacing the terminal adenosine of 3′ end of viral DNA to terminate integration process. 1) Elvitegravir, 2) Raltegravir, 3) dolutegravir, 4) cabotegravir, and 5) bictegravir. The coplanar oxygen atoms are highlighted in red and green circle highlights extended linker a common feature to all second generation INSTIs. MarvinSketch was used to draw and display chemical structures, MarvinSketch v20.11 ChemAxon (http://www.chemaxon.com).

1.4.1.2 Nucleoside reverse transcriptase inhibitors

HIV-1 reverse transcriptase catalyses reverse transcription of viral RNA into complementary DNA. Nucleoside reverse transcriptase inhibitors (NRTIs) halt viral replication by inhibiting reverse transcription. NRTIs bind to a hydrophobic pocket away from RT active site and exert inhibition by competitive inhibition, where modified nucleotide (nucleoside triphosphates) lacking 3′-hydroxyl group on deoxyribose moiety compete with natural deoxynucleotide for incorporation into new strand of DNA (326). Inside the cell, the NRTIs are activated by phosphorylation of deoxyribose moiety by cellular kinase enzymes into active metabolite triphosphates (327–329). When modified deoxynucleotides are incorporated, the incoming deoxynucleotides cannot form 5′-3′ phosphodiester bond required to extend DNA chain (330). The chain termination stops formation of DNA synthesis which halts viral replication (326). The NRTIs have been screened to have highly selective incorporation into the HIV-1 RT over the normal host DNA synthesis to minimize drug toxicity and side effects.

1.4.1.3 Non-nucleoside reverse transcriptase inhibitors

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) do not compete with HIV-1 RT for incorporation of normal deoxynucleotides but bind directly to a pocket near the active site of HIV-1 RT enzyme (41,331). A NNRTI hydrophobic RT complex forms only in the presence of NNRTIs (332,333) and the NNRTIs causes conformational change of substrate-binding site which impairs RT enzyme polymerisation (41,334). The NNRTIs make contacts with hydrophilic residues, K101, K103, S105, D192, E224, and E138; and hydrophobic residues, Y181M Y188, F227, W229, and Y232 (335). The approved NNRTIs for treatment and their abbreviated names in the subsequent text are listed in figure 10.

1.4.1.4 Protease inhibitors

The HIV-1 protease enzyme is relatively small (11 kDa). It is a homodimer of two identical symmetrical subunits of 99 amino acids. It has two mobile flaps which form a cleft, the middle of which is the substrate binding site while the bottom of cleft is the enzyme active site. The

polyproteins Gag-Pol and Gag from translation of mRNA by ribosome are cleaved by HIV-1 protease enzyme into viral enzymes, RT, PR and IN and structural proteins of matrix, capsid, nucleocapsid, p1, p2 and p6 respectively (336,337) as described in sections 1.2.1 and 1.3.8. The protease inhibitors inhibit PR activity through interaction between hydroxyl group of inhibitor and carboxyl group of PR active site residues, Asp 25 and Asp 25' contacting relatively conserved residues Asp 29, Asp 30, Gly 27 and Gly 48 of PR enzyme. This prevents cleavage of Gag-pol and Gag polyproteins which results in formation of immature non-infectious virion particles (338). The PIs are potent regimens with high genetic barrier to resistance but to inhibit fast metabolism and to increase drug's intracellular half-lives, they need to be co-administered with pharmacokinetic enhancers. Ritonavir is a protease inhibitor and also a pharmacokinetic enhancer now commonly used to inhibit cytochrome P450 3A4 isoenzyme to increase the intracellular half-lives of protease inhibitors as well as many drugs used for treatment of various diseases (339,340). Cobicistat (Tybost -GS 9350) is an inhibitor designed to specifically inhibit the cytochrome P450 3A (CYP3A) isoforms including CYP3A4 subtype. Low dose Cobicistat increases systemic exposure and ultimately reduces adverse side effects of atazanavir (ATV), darunavir (DRV), tenofovir (TDF), EVG, and other antiretroviral agents used in treatment of HIV-1. It has also been shown that Cobicistat increases intestinal adsorption of substrates including ATV, DRV and TDF (341). The first PI, saquinavir, was approved by FDA in 1995. The approved PIs for treatment and their abbreviated names in the subsequent text are shown on figure 10.

1.4.1.5 Fusion inhibitors

The HIV-1 envelope consists of surface membrane gp120 and transmembrane protein gp41 which anchors the HIV-1 on to the target cell surface. The binding of gp120 to CD4 receptor triggers disassociation of gp120 from gp41 and gp41 undergoes a conformational change which allows fusion of viral and cellular membranes (342). The essential role of gp41 in anchoring HIV-1 to the cell surface makes it a target for inhibition. The gp41 is predicted to have two hydrophobic helices termed 'heptad repeat' sequences, HR1 and HR2 that are positioned at the base of HIV-1 *env* trimer. The two heptad repeat domains, HR1 and HR2, interact to form a stable six-helical bundle of N helices (interior trimeric coiled coil) and trimeric C helices (342,343) and this interaction between HR1 and HR2 induces membrane fusion (344).

Analysis of gp41 using x-ray structure shows a small pocket in conserved hydrophobic groove of N-peptide bound to isoleucine, tryptophan, and 3 hydrophobic residues which makes it attractive to entry inhibitors (342,345). A non-pocket binding peptide, T-20 is the only fusion inhibitor approved by FDA. T-20 is a synthetic peptide of 36-amino-acid peptide corresponding to C-terminal HR2 sequence of gp41 (181). T-20 disrupts interaction between HR1 and HR2 which disrupts formation of 6-helix bundle required for membrane fusion (181). T-20 has shown potency in patient studies (346,347), and has similar viral inhibition to that of reverse transcriptase and PI (346). However, T-20 is more affected by resistance compared to binding-pocket analogues (348).

Mutations against T-20 are known to reduce viral replication fitness (349,350) and result in hypersensitivity to neutralising of monoclonal antibodies targeting gp41 (349). Resistance mutations against T-20 include, G36DEVS, V38EAMG, Q40H, N43DKS, I37V, N42T, L44M, and L45M (Stanford HIV-1 database, IAS-USA). Resistance mutations to synthetic peptide DP178 early version of T-20 cluster more in first heptad repeat (HR1) than other regions of gp41.

1.4.1.6 HIV-1 coreceptor antagonists

HIV-1 entry into the CD4+ cells requires binding to chemokine receptors CCR5 (178,351) or CXCR4. CCR5 is expressed by lymphocytes, astrocytes, brain microglia, neurons, capillary endothelial cells, epithelium, vascular smooth muscle, fibroblasts, and subpopulations of monocytes (352). The ligands for CCR5 include, macrophage inflammatory protein 1 (MIP-1),α, MIP-1β, RANTES, and HIV-1 gp120 (353,354). CXCR4 is constitutively expressed by lymphatic tissues, spleen, thymus, small intestines and brain (355). The critical role of coreceptors to effective HIV-1 infection is underscored by observation that peripheral blood mononuclear cells (PBMCs) isolated from individuals deficient of CCR5 are not infected by HIV-1 (356–360). Naturally occurring point mutations don't reduce HIV-1 infection (361), but CCR5 missing 32 base pairs (CCR5Δ32) leads to resistance to HIV-1. Individuals homozygous for CCR5 deletion mutation (CCR5Δ32) are resistant to HIV-1 (359,360) and heterozygous individuals for CCR5Δ32 may be protected from numerous abnormalities including, AIDS associated lymphoma (362,363), multiple sclerosis (364–366), rheumatoid arthritis (367,368) and Crohn's disease (369). Individuals with heterozygous CCR5Δ32 also show reduced HIV-1 entry and replication in CD4+

T cells (358). The CCR5Δ32 allele frequencies is around 0%-14% across Eurasia but absent in individuals with Asian or African origin (359,370,371).

CCR5 antagonists bind to a hydrophobic pocket formed by transmembrane helices of CCR5 (372). This allosteric interaction results into locks the receptor into a conformation that is unrecognisable by HIV-1 Env (373). There are four CCR5 analogues which have shown potency as CCR5 antagonists; maraviroc, aplaviroc, vicriviroc, and leronlimab (PRO 140). Maraviroc was approved in 2007 (374,375), Vicriviroc was not filed for FDA approval following a successful phase III clinical trial, Aplaviroc was discontinued due to severe liver toxicities (376), and Leronlimab is a monoclonal antibody that binds to CCR5 receptor on the CD4+ T cells that has emerged as treatment following clinical trials.

Maraviroc is recommended in treatment experienced patients infected with CCR5 usage viruses. Use of CCR5 antagonist in patients infected with CCR5/CXCR4 dual tropic viruses may encourage emergence of CXCR4 virus and rapid virological failure. Therefore, it becomes paramount to screen patients with HIV-1 showing even minimal usage of the CXCR4 coreceptor before prescription of MVC, a CCR5 antagonist. Resistance to CCR5 antagonists is mainly through 3 mechanisms; ability of HIV-1 Env to bind CCR5 bound to MVC (377,378), selection of CXCR4 by originally CCR5 viruses (379,380), and increased affinity of CD4 resulting in HIV-1 remain bound to cell surface in a confirmation to engage CCR5 when MVC dissociates (based on its on-off binding). Generally, most mutations against CCR5 occur in V3 loop of gp120 (Maraviroc Viiv healthcare) though resistance mutations have also been observed in gp41 (381). Serial passage assays show selection of resistant mutations, T316A, V323I in V3 loop, contrary to resistant mutations against aplaviroc which preferentially cluster in C1-5, V1, V3 of gp120 and in gp41 protein. The CCR5 antagonist functions by binding to CCR5 which leads to conformational change in coreceptor, in particular, the second extracellular loop (ECL2) which terminates binding to V3 loop of HIV-1 gp120 (382,383). Some inhibitors like aplaviroc bind directly to ECL2. The CXCR4 antagonists halt HIV-1 entry by inhibiting interaction between CD4-gp120 complex with ECL2 region of CXCR4. Resistance emerging through enhanced CD4 interactions is conferred by the N425K mutation in the CD4 binding pocket in gp120. This mutation provides a gain in replicative fitness but never emerges in patients with or without MVC treatment which may relate to exposing gp120 epitope inducing neutralizing antibodies which in turn eliminate this mutated virus (384).

1.5 HIV-1 drug resistance

HIV-1 drug resistance (HIVDR) is characterized by active viral replication in presence of ART, and it is the major reason for treatment failure (385). All currently available drugs for treatment of HIV-1 can select for HIVDR with first generation inhibitors more susceptible to HIVDR compared to newer generation of inhibitors. There are a number of factors which can lead to development of HIVDR; poor drug levels in patients taking the prescription but subtherapeutic blood drug levels is related to poor drug absorption, drug-drug interaction or possible genetic polymorphisms impacting drug metabolism. Emergence of drug resistance is rare in patients adherent to the drug regimen and the latter factors appear a low frequency in the patient population. Instead, HIVDR is primarily related to poor treatment adherence (Fig. 14) due to fatigue in maintaining life-long daily treatment, drug stock outs, taking "holidays" from drug treatment, avoiding the stigma or hiding treatments from others, and taking wrong dosages.

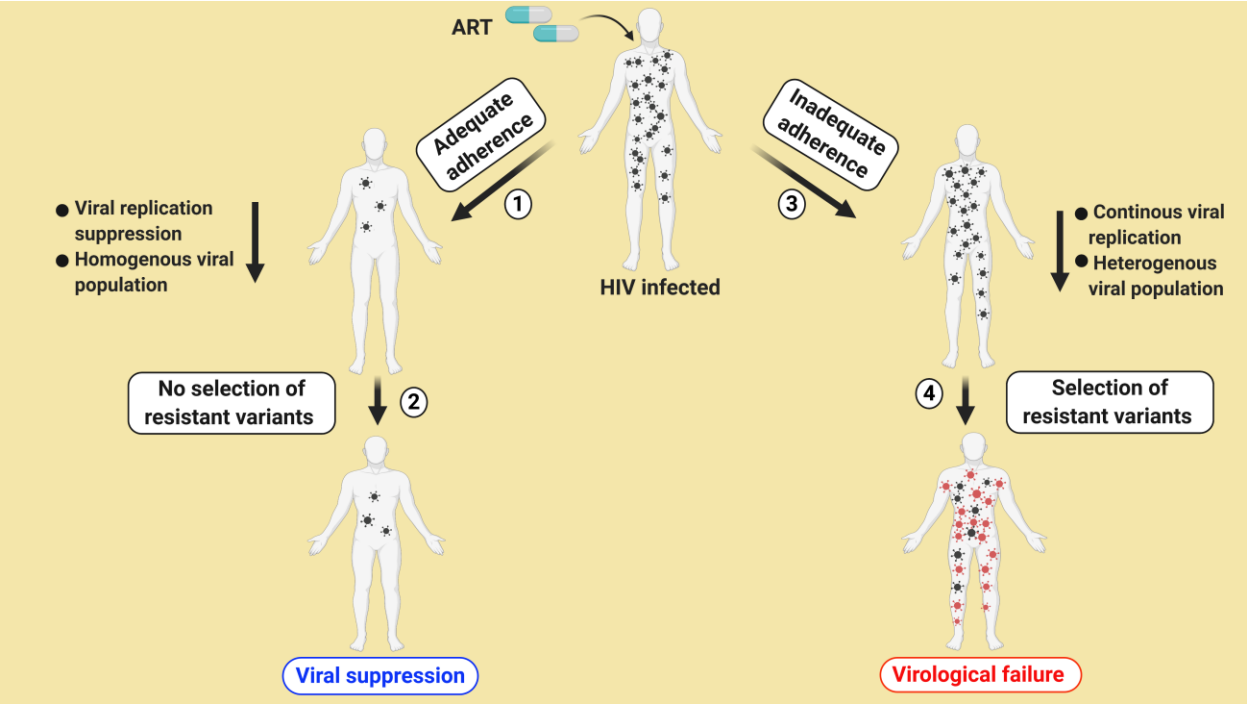

Figure 14. The role of patient adherence to ART. 1) Adequate adherence to ART (>90%) allows viral suppression within first 3 months of starting ART in most patients. 2) Suppressed viral load inhibits selection of drug resistant variants in presence of ART. 3) Patients starting ART and not adequately adhering to ART (<90%) experience immunodeficiency rapid viral replication. 4) High

viral replication in presence of suboptimal drug dosage leads to preferential selection drug resistant variants in presence of ART.

The HIV-1 mechanism(s) behind the emergence of drug resistance is rather simple compared to the sociodemographic and physiological factors that set the stage for treatment failure. Compared to all other RNA and DNA polymerases in nature, HIV-1 RT has one of the highest misincorporation rates of nucleotides and does not possess repair mechanism such that replication of each HIV-1 genome can have been 1 and 10 mutations. With a turnover rate of approximately a billion HIV-1 particles/day within an infected individual, nearly every possible mutation encoding for a replication competent virus emerges every day. The survival of each mutant HIV-1 strain forming a swarm of clones or "quasispecies" is based on the replicative fitness of each strain. Under drug selective pressure, the strain with a specific mutation conferring reduced susceptibility to the drug is immediately selected in the population. In general, the current generation of antiretroviral drugs select for HIV-1 mutants with low replicative fitness and of very low frequencies in the quasispecies of a patient. Alternatively, the mutations conferring resistance are lower in the "fitness valley" and require multiple compensatory mutations to emerge for effective drug resistance and treatment failure.

1.5.1 Mode of resistance to HIV-1 inhibitors

1.5.1.1 Resistance to entry inhibitors

Though sparsely used compared to other classes of ART, the clinical use of entry inhibitors has led to emergence of resistance to these inhibitors mainly through a coreceptor shift from CCR5 to CXCR4, and changes in the viral envelope which allows viral replication to continue in presence of inhibitors. HIV-1 resistance mutations against CCR5 antagonists are scattered in different regions of gp120 including V2, V3, C2 and C4 (386–388). Resistance mutations to many CXCR4 antagonists have been observed on V3 domain of gp120; however, mutations against SDF-1α analogue in V1, V2, C2, and 5 amino acids deletion in V4 loop of gp120 have also been observed in resistance selection assay (389). No coreceptor usage switch from CXCR4 to CCR5 been observed with CXCR4 antagonists (389).

1.5.1.2 Resistance to NRTIs

Resistance to NRTIs mainly occurs in two ways; discrimination and excision pathways (326). During discrimination pathway, the HIV-1 RT enzyme affinity to bind to NRTIs is decreased due to mutations in the N-terminal domain of polymerase while retaining the ability to recognise natural deoxynucleoside triphosphate (dNTP) substrate e.g. RT mutations M184V and V75T. In another form of discrimination, there is a decrease in rate of incorporation of analogue over natural nucleotides, e.g. K65R, K70E, L74V, and Q151M. In the second mechanism to NRTIs resistance, the incorporated chain terminating analogues get excised from the 3′-terminus after it has been added into the viral DNA through pyrophospholysis. This allows DNA chain elongation to continue resulting in viral replication. The mutations may affect phosphorolytic activity of RT overcoming activity of NRTIs in a process called primer unblocking. The mutations which enhance loss of phosphorolytic activity of RT include those against thymidine analogues, AZT and d4T (stavudine), exhibit this form of antagonism; D67N, K70R, L210W, T215Y/F, K219E/Q.

1.5.1.3 Resistance to NNRTIs

The NNRTIs inhibit viral replication by non-competitive inhibition; where analogues bind the p66 subunit of HIV-1 RT at a hydrophobic pocket near active site of RT which terminates elongation of DNA chain. The mutations result in conformational changes of the binding pocket through disruption of specific contacts between inhibitor and binding pocket, e.g. K103N, K101N; disruption of interactions inside pocket e.g. Y181C, Y188L; and overall conformational change of pocket e.g. G190E which makes a steric bulk in the pocket leaving no space for NNRTI binding. NNRTIs mutations, K103N against nevirapine (NVP), and efavirenz (EFV); and Y181C, against NVP, EFV, rilpivirine (RPV), and etravirine (ETR) are the most commonly selected in treated patients (390–392). HIV-2 and half the isolates of HIV-1 group O are intrinsically resistant to most NNRTIs (393). The drug resistance mutations observed in patients failing NNRTIs regimen all reside in the NNRTI binding pocket. NNRTIs have better tolerability profiles than NRTIs, but have lower genetic barrier to resistance since a single mutation in HIV-1 RT hydrophobic pocket confers high resistance to most NNRTIs with minimal or no impact on replicative fitness. Resistance to NNRTIs usually emerges rapidly in case of incomplete viral suppression or when

administered as monotherapy which suggests that pre-existing mutations are quickly selected under NNRTIs pressure (394–397). Again, since mutations conferring NNRTI resistance don't cause substantial loss to viral replication fitness (392,398), NNRTI resistant HIV-1 remain detectable in patient's viral population many years after stopping a failing NNRTI-based treatment regimen. Non-subtype B viruses may be intrinsically more resistant to NNRTIs due to pre-existing background polymorphisms (399,400). Paradoxically, NRTIs resistance mutations for example, at codons 118, 208, and 215 were shown to cause hypersensitivity to NVP, EFV and DLV (401).

1.5.1.4 Resistance to PIs

The symmetrical structure of HIV-1 PR is crucial for efficient binding of PIs into the substrate-binding site. The resistance to PIs is a result of mutations near the substrate-binding cleft of an enzyme which cause a conformational change, thus preventing the binding of PIs directly or indirectly leading to resistance (402–405). Most primary PI resistance mutations (e.g. V82A, I50V, G48V, I84V) are located near the enzyme active site. The mutations in the substrate-binding site of PR enzyme also affect the binding of normal substrates-Gag-pol and Gag polyproteins which affects the normal processing of immature proteins, resulting in impaired viral replication capacity (406–408). The loss in viral replication capacity (RC) is usually compensated by emergence of compensatory mutations which increase resistance to PIs (409–411) and restores the RC (410–412). Thus, mutations in the PR cleavage sites within the Gag and Gag-Pol precursor proteins may act as compensatory mutations to ensure normal PR-mediated processing and HIV-1 core formation (337,408,413–415). The resistance mutation against one particular PI usually affects nearly all other first-generation PIs due to similar chemical structures and the substrate binding site in PR.

Mutations in HIV-1 Gag located in close proximity to, but not within the canonical seven amino acids of cleavage sites have been associated with evolution of HIV-1 PR resistance mutations, for instance mutation I47V downstream of the NC/sp2 cleavage site, and P453L downstream of the sp2/p6 site (416,417). The mutations in HIV-1 Gag cleavage sites have also been shown to reduce susceptibility to HIV-1 PIs. The presence of NC/p1 cleavage site substitutions, K436E and/or I437T/V conferred resistance to LPV (lopinavir), ATV, tipranavir and amprenavir *in vitro* (418).

1.5.1.5 Resistance to INSTIs

Resistance to INSTIs occurs mainly through mechanisms involving conformational change of HIV-1 IN affecting the binding of INSTIs, charge effects, loss of stabilising IN and INSTIs interactions, and steric hindrance. The residues involved in INSTIs resistance are highly conserved in HIV-1 IN protein. The common resistance pathways to RAL and EVG observed *in vivo* and *in vitro* are commonly seen at positions N155, Q148, Y143, and E138 of HIV-1 IN (Fig. 15). Second generation INSTIs, DTG and BIC are commonly associated with mutations at positions R263 and Q148 in combination with other mutations. Resistance mutations at position Q148 are also associated with CAB, the long-acting derivative of DTG. Most of the residues involved in resistance are in proximity of surface α4 helix of CCD (Fig. 15) and some make direct contact with INSTIs. Thus, the binding of INSTIs to the HIV-1 IN-DNA complex and crucial connections by these residues within the complex may explain the occurrence of these mutations in HIV-1 IN.

HIV-1 drug resistance to RAL portrays three major mutation pathways, Y143R/C, Q148R/K/H and N155H (419). Mutations, N155H and Q148H/K/R confer 10-fold and 25-fold resistance to RAL respectively (420). Other mutations against RAL observed *in vivo* include: L74M, E92Q, E138K, G140S/A and G163R (420). The mutation Y143H/C/R is specific to RAL (421,422) and F121Y is selected by RAL only *in-vitro.* Drug resistance to EVG occurs mainly through primary mutations, T66A/I/K, E92Q, N155H, S147G, and Q148H/K/R and G140S/A/C (423–425). Mutations S147G confers resistance exclusively to EVG. Secondary mutations conferring resistance to EVG include: H51Y, Q195K, Q146P, E138K/A/T, and G118R, (425,426) with F121Y, P145S, Q146P, and R263K reducing EVG susceptibility *in vitro*. Mutations T66I and E92Q reduce susceptibility to EVG by >30-fold and Q146P, S147G, H51Y, Q95K, and E157Q by <10-fold (426).

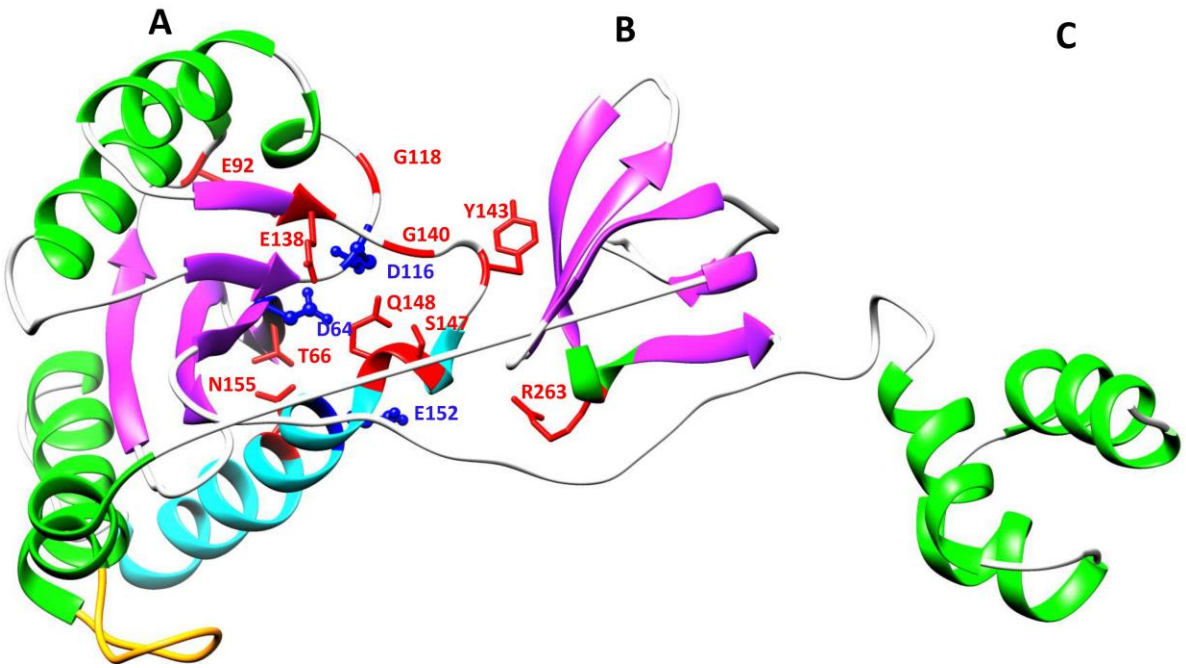

Figure 15.The common INSTIs DRMs in different domains of HIV-1 IN. A) In the CCD, the majority of INSTIs associated DRMs (red) are observed in proximity to the three catalytic triad residues of IN active site (blue) and surface α4 helix (cyan). B) Residue R263 in CTD that is commonly associated with DTG resistance. C) Currently no INSTIs associated mutations been observed in NTD of HIV-1 IN.

Mutations Y143R/C, Q148R/K/H and N155H are located within the CCD and in close juxtaposition to the catalytic triad of D64, D116 and E152 in CCD. Though Y143 lies slightly outside the catalytic pocket, Q148 is located close to the DDE residues and N155 is surrounded by D64 and E152 residues. In the co-crystal structures, there is no direct contact observed between residues Q148 and N155 with bound INSTIs (Fig. 12). However, owing to their critical position in active site, substitutions at these positions may trigger a conformational change within the catalytic pocket which impairs INSTIs binding (310). The α4 helix residues Y143 and Q148 make crosslinks with 3-processing site CA GT3'/5' ACTG of U5 and U3 of HIV-1 LTR and may confer resistance by facilitating accurate positioning of target and vDNA substrates. The small and flexible amino acid G140 has been shown to accommodate movement of the His side chain at position Q148 enabling INSTIs to bind with a less significant conformation change of IN. This probably explains the increased resistance seen with Q148H+G140S/A compared to single Q148H (310). Further still, the presence of bulky Lys or Arg in Q148K/R disrupts the active site which

impairs binding of INSTIs. Disruption of the His-phosphate interaction by INSTIs may explain resistance at residue N155 (310). The resistance associated with Y143 may be due to direct interaction with the oxadiazole group and phenol ring of bound RAL forming face-to-face π π stacking interaction that is abrogated when this residue is mutated (310,427). INSTIs, EVG, DTG, BIC and CAB which don't have an oxadiazole group are not much affected by substitutions at Y143 position. The residues E92 and F121 are located close to bound EVG which may explain resistance at these positions. The primary resistance mutations to EVG are scattered around the catalytic triad surrounded by secondary mutations. Residues G118 and S153 associated with DTG resistance, are in close proximity to catalytic triad residues D116 and E152 respectively. G118R may confer resistance by altering geometry of catalytic triad affecting INSTI chelation of divalent cations in active site (428) or through causing steric hindrance to drug binding due to bulky arginine compared to glycine residue (308). R263K is located far from active site and reduce DTG susceptibility by reducing on vDNA binding (429).

Contrary to EVG and RAL, DTG possess a higher resistance barrier (430,431) and is generally less affected by mutations known to cause cross resistance in RAL and EVG (432,433). DTG selects a rare R263K mutation *in vitro* (434) and *in vivo* (435) but at a cost of reduced RC (319,429,435) and ultimately impaired infectivity (436). Other resistance mutations associated with resistance to DTG have also been found in both patients failing EVG and RAL-based treatments and *in vitro* assays at positions F121, S153, G118, and E138 (319,429).

Resistance mutations R263K, G118R, H51Y and E138K confer low level resistance to DTG *in vitro* (429,437). These mutations alone or combined with secondary mutations do impair RC. A combination of R263K and mutations at positions 143 and 148, leads to a drastic reduction of enzyme activity (434). R263K can tolerate the presence of two mutations, E92Q and N155H of all other mutations associated with RAL and EVG (436). In the presence of R263K or G118R and secondary mutations, H51Y, M50I, and E138K, the RC is significantly impaired or not restored (434,438,439). R263K mutation has influence also on accessory mutations to N155H pathway under DTG pressure. Despite viruses containing N155H and R263K in presence of DTG causing low level resistance against DTG, surprisingly, no change in resistance level against DTG is observed when R263K/N155H is added to L74M, E92Q, T97A, E157Q and G163R, common secondary mutations to N155H in patients failing RAL/EVG treatment (440). A combination of

S153F/R263K mutations shows >100-fold resistance to DTG, RAL, EVG, BIC, and CAB *in vitro* (441). However, emergence of S153F and R263K as minority variants does not seem to translate into virological failure in a patient on DTG treatment over 24 weeks (441).

Clinical trials, SINGLE (DTG/3TC/abacavir (ABC) vs TDF/FTC/EFV) (442), FLAMINGO (once daily DTG vs ritonavir boosted DRV)(443), and SPRING-1 and 2 ([once daily DTG versus EFV and twice-daily RAL respectively] drugs given with coformulated TDF/FTC or ABC/3TC) (444) assessed efficacy of DTG-based regimens in ART naïve patients, and no emerging DTG resistance was observed in patients given DTG-based regimens. In highly ART experienced patients with IN resistant virus to RAL and EVG, DTG (50mg) twice daily showed efficacy with 69% of patients achieving HIV RNA <50 copies/ml at week 24 but Q148HRK+ $\geq$ 2 mutations reduced odds of achieving HIV-1 RNA <50 copies/mL by 96% (318). In a study which compared once daily DTG versus twice daily RAL in ART experienced but INSTIs naïve patients (SAILING study), no emerging resistance mutations with genotypic and phenotypic resistance to RAL or DTG was observed in DTG arm but RAL-associated genotypic resistance emerged in 42% patients with virological failure taking twice daily 400mg RAL (435). Nevertheless, there are case reports and clinical studies reporting cases of DTG resistance. A case report shows virologic failure in an ART naïve patient on DTG/FTC/TDF and rifampicin and failure was associated with reduced DTG drug levels based on interactions with rifampicin (445). In this patient, virologic failure coincided with the emergence of R263K and E157Q in IN and the M184I in RT, i.e. conferring resistance to FTC (445). Another case report reported a patient on TDF/3TC/DTG regimen who had virologic failure associated with I151V, G163R, and Q148K in IN and M184V in RT (446). In ART experienced but INSTIs naïve patients in the SAILING study and a retrospective cohort study (DTG vs RAL vs EVG), DTG resistance has been observed with IN T66I and R263K mutations emerging DTG-based arm (435,447). In HIV-1 infected patients, high RNA copies >100,000 copies/ml, CD4 T cell of < 200 cells/ul, adherence <80% (416), and coinfection with *Mycobacterium tuberculosis* (420), have been shown to be risk factors to emergence of DTG resistance. Despite these anecdotal cases, emergence of DTG resistance is rare following initiation of a new DTG-based cART among treatment experience patients and extremely rare in treatment naïve taking DTG-based cART. These observations, the high genetic barrier for DTG resistance, and the low replicative fitness of DTG-resistant HIV-1 prompted the field to consider use of DTG in monotherapy. DTG resistance rapidly emerged in ART experienced, virologically suppressed patients (who were INSTI naïve)

when switched to DTG monotherapy. DTG resistance was conferred by Q148H/R (3-patients), N155H (3-patients), G118R (2-patients), S230R (2-patients) and R263K (1-patient) (448,449).

Interestingly, INSTIs mutations against DTG may have an inhibitory effect on NRTIs and NNRTIs mutations. The presence of H51Y/R263K delays the emergence of NNRTI resistance mutation, V106A by 5 weeks while R263K delays NRTI resistance mutation, M184V/I by 2 weeks (450). Emergence of NNRTI resistance (Y181C) required more viral passages and a delay between 15 to 25 weeks when comparing wild type HIV-1 versus HIV-1 harboring INSTI resistance mutations G118R, H51Y/G118R (450). The emergence of V106A mutation was delayed from week 6 to 25 weeks in viruses carrying H51Y/R263K vs wild type in presence of NVP. With 3TC, there was delay in emergence of M184I to week 25 in presence of H51Y/R263K mutations (450). In addition, presence of the INSTI-resistance mutations, R263K with either the NRTI resistance mutations, M184V/I or K65R mutations further reduce viral replicative capacity of the viruses with any one of these mutations alone (451). The R263K mutation progressively reduces HIV-1 integration (452) and there is no detectable levels of integrated HIV-1 DNA in cell culture assays in presence of R263K/H51Y and M184I/V, L74V, K103N, E138K (451). Together, these observations may explain why cases of DTG failure with NRTI resistance mutation is rare, i.e. DTG resistant mutations cannot emerge or are slow to emerge due to additive reduction in replicative fitness. These observations also indicate impaired capacity of HIV-1 to develop resistance mutations in different genes at the same time under drug pressure. Combination of mutations in RT and IN genes also seem to interfere with interaction between these two genes leading to reduced RC. HIV-1 IN mutations being pleiotropic, they have been described upon multiple viral steps including reverse transcription. HIV-1 INSTI drug resistant mutations have been found in all three domains of HIV-1 IN enzyme (453) and disruption of binding residues of RT on HIV-1 IN led to downregulation in synthesis of cDNA (454). Interestingly, presence of RT mutations M184I/V or K65R impair the emergence of DRMs against DTG but not EVG or RAL (455).

BIC has resistance pathway involving R263K, S153F/F, H51Y, Q146L in cell culture (456,457), and Q148H. BIC generally retains activity against mutations T97A, T66I, E92G/Q, Y143R, Q148H/K/R, Q148H/G140S, and N155H, conferring resistance to EVG, RAL (458,459), and DTG associated mutations, G118R, R263K, H51Y/R263K (459). In phase 3 clinical trials investing efficacy of single-tablet regimen BIC/FTC/TAF compared to DTG regimens in ART naïve

patients, no emerging resistance to BIC was observed in any of the study patients (460,461). In GS-US-380-4030 study investigating efficacy of single-tablet regimen BIC/FTC/TAF in virologically suppressed patients with documented or suspected NRTI resistance, no emerging resistance to BIC was reported in the study (462).

CAB an analog of DTG shows activity in presence of RAL, EVG, and DTG mutations, Y143R, N155H, T66I, E92Q, H51Y and E138K/R263K with EC_{50} of <5 nM (458), and Q148H/K/R, G140S/Q148H/K/R, (FC <7) (463). The oral formulation of CAB demonstrated a higher genetic barrier to resistance than RAL and EVG (absence of mutations at residues, 138, 140, 148, and 155) in a first time-in-human and phase IIa study involving healthy and HIV infected individuals (464). The long-acting CAB has also shown activity *in vitro* in viruses from ART naïve patients (mean EC_{50} FC, 0.91) and those harboring dual N155H/E92Q (4-fold) and Q148H/G140S (11-fold) mutations in a panel of subtype B, A1, D, C, and recombinant AE recombinant viruses (465). In a randomized, phase 2b, open-label clinical trial of ART naïve patients investigating intramuscular CAB, resistance mutation Q148R emerged in one of the three patients who experienced virological failure (286), and Q148R has also been observed in patient failing -oral CAB (325). So far, long-acting injectable CAB has been tested in two phase III clinical trials, FLAIR and ATLAS. In these trials investigating non-inferiority of monthly CAB+RPV compared to current oral regimens, there was no emergence of resistance FLAIR study (466) and N155H mutation which reduced CAB susceptibility by 2.7-fold was observed in ATLAS study (289). In LATTE-2 trial comparing infectable CAB/RPV and oral CAB/3TC/ABC, R269R/G and Q148R emerged in 2 patients with protocol-defined virological failure in CAB/RPV arm and only Q148R showed phenotypic resistance to CAB (286). Overall, BIC, CAB, and DTG have higher genetic barrier to resistance than EVG and RAL, but DTG and BIC show broader efficacy against resistant viruses compared to CAB (457,459).

1.6 HIV-1 subtypes and recombinants

The HIV-1 epidemic is a result of zoonotic viral transmission of SIV from nonhuman primates to humans mainly in regions of Western and Central Africa. Transmission from lentiviral strains, SIVcpz (chimpanzees) and SIVgor (gorilla) resulted in HIV-1 groups M&N and groups O&P, respectively. With HIV-1 groups O and P, SIVgor may have passed into chimpanzees prior to the

jump to humans. HIV-2 originated from SIVsmm (Sooty mangabey). The high error prone HIV-1 RT and high turnover of HIV-1 virions within infected humans ultimately resulted into high genetic diversity of HIV-1 circulating worldwide. HIV-1 is mainly divided into 4 main groups, group M (major), O (Outlier), N (non M, non-O) and N (11). Group M is the most widely distributed and is further divided into 9 subtypes: A-D, F-H, J and K (11). Globally, subtype C accounts to the majority of HIV-1 infections (48%), subtype A (12%), subtype B (11%), CRF02_AG (8%), CRF01_AE (5%), G (5%) and D (2%) of HIV-1 infections (467). HIV-1 subtype A is predominant in regions of Eastern Africa (468), Asia and other former Soviet Union countries (469). HIV-1 subtype C is commonly found in Southern Africa, Eastern Africa, Brazil, China, and India and has been increasing in regions of Brazil, Southwest China, Malaysia, Scotland, and Uruguay. HIV-1 subtype B predominates in regions of North America (470), South America (471), Australia (472), Europe (473), Middle East and Northern Africa (474). Subtype F is mainly in regions of Eastern Europe and southern America. Subtypes E, G, H, J, and K are mainly in countries of Central and West Africa. It is important to note that all of these HIV-1 subtypes had their origins in the Congo Basin, likely due to an unreported emergence of HIV-1 infections following the "opening" of this tropical rainforest region with Colonist expansion into this region following the turn of the 19th century.

HIV-1 like all retroviruses has two copies of genomic RNA (diploid) on which RT can jump between strands during reverse transcription to complete the proviral DNA (see section 1.3.3). In cases of dual or super infection by two different HIV-1 strains within an individual human, these two HIV-1 strains can dually infect a cell and produce a heterodiploid virus (a copy of an RNA genome from both strains) which can then lead to recombinant HIV-1. Thus co-circulation of different HIV-1 subtypes in the same geographical region will eventually lead to stable circulating and unique recombinant forms (CRFs and URFs). A strain is recognized as CRF if it is isolated from at least two unrelated individuals, fully sequenced and plays a role in HIV-1 pandemic. The unique recombinant form (URFs) result from a mixture of subtypes found only in one HIV-1 infected individual (475). Approximately 20% of group M viruses found in the epidemic are not "pure" subtypes but are recombinant forms (476). These recombinations are widely distributed in regions of Western Africa and Asia with 101 CRFs identified as of 2020 (https://www.HIV-1.lanl.gov/content/sequence/HIV-1/CRFs/CRFs.html).

Originally known as subtype E, CRF01_AE, has subtype A genes and subtype E *env*, circulates in South-East Asia and accounts to >80% HIV-1 infections in Thailand and 5% globally. CRF01_AE/B is mainly found in Thailand, Georgia, and Malaysia. The CRF02_AG, which is commonly found in Western Africa, accounts for 60% HIV-1 infections in Cameroon and nearly 5% of global HIV-1 infections. The former subtype I now classified as the CRF04_cpx, a subtype A/G/H/K recombinant (477,478)(479), accounts for 2-10% of HIV-1 infection in Greece (480), Cyprus and Mediterranean regions. CRF06_cpx from parental fragments of subtypes A, G, J, and K is responsible for infections mainly in Burkina Faso and Mali; and CRF07_BC, and CRF08_BC are the dominant HIV-1 forms in China (12, 13-Bertha 2008). CRF09_cpx from subtypes A, C and D accounts for most HIV-1 infections in Senegal. Intermixed recombinant forms have also been isolated with CRF18_cpx/CRF19_cpx isolated in Cuba, CRF06_cpx/A in Estonia and CRF11_cpx/B in Switzerland.

The high degree of diversity between group M and O has not hindered occurrence of homologous recombination between the two groups with patients carrying intergroup M and O replicative competent (481). Recombination between HIV-1 and HIV-2 types has not yet been documented probably because of wide diversity between the two groups, however, patients infected with HIV-2 can subsequently be infected with HIV-1 (482).

HIV-1 Groups O, N, and P all appeared within or in the vicinity of Cameroon and never attained the same foothold in the human population as HIV-1 group M. There is less than reported infections with N and P while HIV-1 group O is responsible for ~50,000 infections worldwide. HIV-2 strain shows low infectivity characterized by low viral loads and slow disease progression in humans and this may explain its limited circulation/expansion in areas of Western Africa.

1.6.1 HIV-1 subtypes and drug resistance

Patients respond similarly to ART treatment irrespective of the HIV-1 subtype (483–485), however, HIV-1 subtype may still influence ART outcomes and emergence of HIVDR. For instance, despite susceptibility of HIV-1 subtype C and B strain-derived IN enzymes to the current approved INSTIs, there is different treatment outcomes in patients infected with subtypes B and C

infections on INSTIs (486). There is also less chance of subtype B viruses developing resistance against DTG compared to subtype A/G and C viruses (429). HIVDR against NRTIs and PIs has been shown to occur more frequent in subtype B compared to subtype C (26% vs 8% and 54% vs 23%) respectively. Faster emergence of HIVDR to NRTIs and PIs in subtype B vs C has been shown in ART naïve and experienced patients (487). In Uganda, ART drug resistance and treatment failure is more prevalent in subtype D compared to subtype A or subtype C infected patients (488). The variability of INs at amino acid level between different HIV-1 subtypes is relatively low ~8-12% however, higher entropy scores or amino acid variability is observed at sites where mutations are selected for primary or secondary INSTI resistance.

Subtype-specific resistance mutations to INSTIs: Among HIV-1 subtypes, residues associated with IN catalytic activity and common INSTIs mutations sites, N155, Q148, Y143 are highly conserved, however there is variability at secondary resistance mutations sites among different HIV-1 subtypes (489) (Fig. 16). HIV-1 subtype specific amino acid differences have been observed near primary or secondary resistance mutations (490).

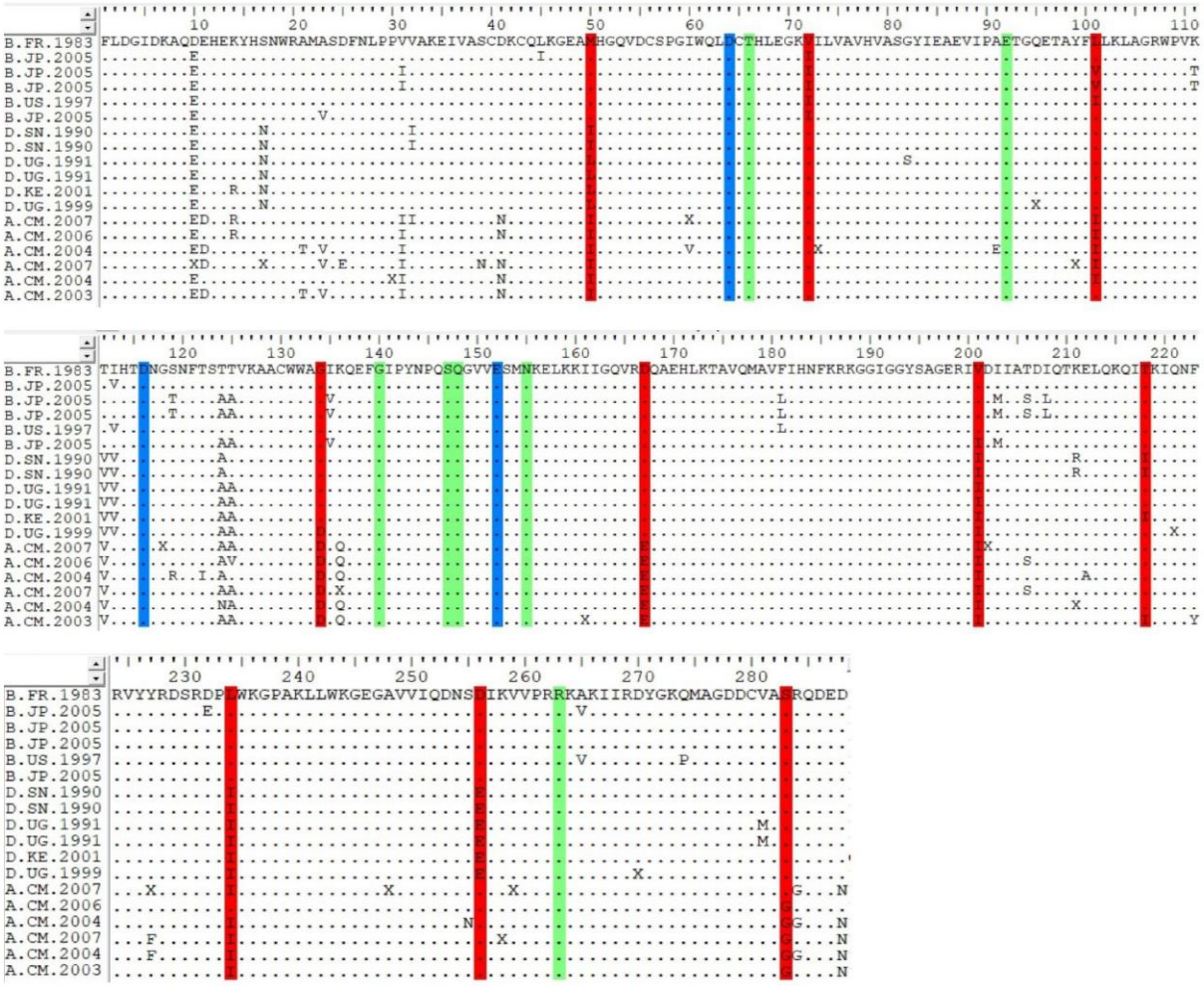

Figure 16. HIV-1 IN of different subtypes. Across HIV-1 integrase gene there are a number of differences in naturally occurring polymorphisms in different HIV-1 subtypes. These polymorphisms may function as compensatory mutations in specific subtypes which may influence differential selection of major mutations. HIV-1 integrase sequences of ART naïve patients were downloaded from HIV-1 sequence database (http://www.HIV-1.lanl.gov/). Alignment, annotation, and visualization was done in Bioedit (491). Blue represents the three catalytic triad residues of CCD (D64, D116, and E152), green represents major resistance mutation positions, and red is the natural polymorphisms naturally occurring between different HIV-1 subtypes. General consensus of amino acids at a particular position between HIV-1 subtypes is represented by (.).

HIV-1 mutations at position G140 of HIV-1 IN occur more frequently in subtype B than non-subtype B infected individuals (492) which also increases the frequency of Q148H/K/R mutations in subtype B viruses (Table 1). This is attributed to the high genetic barrier to the emergence of G140A/S in non-subtypes compared to subtype B. In subtype B virus, glycine at position 140 is

coded commonly by GGT or GGC thus requiring a single transition G to A to replace glycine with serine (G140S) or transversion G to C for the alanine (G140A). In contrast, glycine is commonly coded by either GGG or GGA in non-B subtypes requires two nucleotide substitutions to change to codon G140 mutants. In cell culture assays, R263K mutation selected by DTG and EVG is frequent in HIV-1 subtype B and CRF02_AG, whereas G118R conferring resistance to DTG, RAL and EVG is common in CRF02 A/G and C subtypes (429). Whereas G118R causes low-level resistance to RAL in subtypes B and C, there is increased resistance to RAL by G118R in CRF02_AG in cell culture and patient samples (437,493). E138K mutation which confers resistance to RAL, EVG, and DTG in presence of Q148 mutations shows increased level resistance to RAL in CRF02_AG compared subtype B viruses (437) (Table 1). Double mutation E92Q/N155H in HIV-1 subtype C backbone shows 10-fold more susceptibility to RAL and EVG compared to the same mutations in subtype B virus (486). On effect of subtype on IN enzyme activity, H51Y causes significant loss in IN enzyme activity in CRF02_AG and B but not in subtype C, and E138K leads to >55% reduction in enzyme activity in CRF02-AG but not in subtype B and C (437). Strand transfer efficiency is restored by addition of E138K+H51Y to G118R in subtype B but not as much with subtype C and CRF02_AG (437).

Table 1. The INSTIs associated resistance mutations frequency and impact on susceptibility in different HIV-1 subtypes

HIV-1 mutation	Cell culture/patients	Mutation frequency		Reference
		a) Increased frequency	Less frequency	
R263K	Cell culture	B	CRF02_AG, C	(425)
G118R	Cell culture	Subtype C and CRF02_AG	CRF02_AG	(425)
G140S/A	ART naïve /experienced	B	Non-B subtypes[a]	(486)
Q148H/R/K	ART naïve /experienced	B	Non-B subtypes[a]	(486)
E92Q, Q148H/R	ART naïve /experienced	B	Non-B subtypes[c]	(487)
L74M/A/I	ART naïve /experienced	Non-B subtypes[b]	B	(488)
T97A	ART naïve /experienced	Non-B subtypes[b]	B	(488)
V165I	ART naïve /experienced	Non-B subtypes[b]	B	(488)
V201I	ART naïve /experienced	B	Non-B subtypes[b]	(488)
T206S	ART naïve /experienced	B	Non-B subtypes[b]	(488)
K156N	ART naïve /experienced	B	Non-B subtypes[b]	(488)
I203M	ART naïve /experienced	B	Non-B subtypes[b]	(488)
V72I	ART naïve	B	CRF01_AE	(489)
L10I	ART naïve	B	CRF01_AE	(489)
A124T	ART naïve	B	CRF01_AE	(489)
T125K	ART naïve	B	CRF01_AE	(489)
G140C/S	ART naïve	B, A	CRF01_AE, CRF02_AG, C	(489–491)
V201I	ART naïve	CRF01_AE	B	(489)
L74M	INSTIs naïve	CRF43_02G	B, C, D and CRF01_AE	(492)
T97A	INSTIs naïve	Group P, J, CRF18_cpx and F2	B, C, D and CRF01_AE	(492)
G163RK	INSTIs naïve	CRF44_BF, CRF46_BF, CRF17_BF, F1, CRF12_BF and CRF29_BF	B, C, D and CRF01_AE	(492)
V151I	ART naïve	B, C	CRF02_AG, A/ CRF01_AE	(490,491),
L10I	ART naïve/experienced	C, F, CRF02_AG	D, CRF01_AE	(487)
T124A	ART naïve	D, CRF01_AE, CRF02_AG	B and F	(487)
G193E	ART naïve/experienced	B and F	C, D, CRF01_AE, CRF02_AG	(487)
L101I + T124A	ART naïve	CRF02 AG, and C	B and D	(487)

		b) Increased drug resistance	Reduced drug resistance	
E92Q/N155H	Cell culture	B (RAL and EVG)	C	(478)
G118R	RAL-experienced	CRF02_AG (RAL and EVG)	B	(485)
Q148R, N155H	Cell culture	HIV-O (DTG)	B	(493)
G98, E179, C181, R103, T215	Cell culture	HIV-O (EVG)	HIV-M	(494)
G118R	Cell culture	CRF02_AG (RAL)	B and C	(433)
E138K/Q148	Cell culture	CRF02_AG (RAL)	B	(433)

[a] A, C, D, F, G, CRF01, CRF02, CRF01, CRF05, CRF35; [b] A, C , D, F, G, H, J, CRF01_AE, CRF02_AG, CRF03, CRF06, CRF09, CRF11, CRF12_BF, CRF13, CRF14_BG, CRF15, CRF18, CRF19; [c] A, C, D, F, G, CRF01_AE, CRF02_AG. In bold, major INSTIs DRMs. In brackets, INSTIs for which there is increased resistance in presence of respective DRMs.

Subtype-specific resistance mutations to PIs: Natural polymorphisms, M36I, V82I, L10I, L63P, L10V, K20R, and V77I in protease gene of non-subtype HIV-1 in ART naïve patients are known to reduce susceptibility to PIs in HIV-1 subtype B viruses (487,494). Substitutions at position 36 of HIV-1 protease seem to have differential impact on susceptibility of ART in different subtypes. I36 appears as an accessory mutation and enhances resistance to ritonavir, ATV, and nelfinavir as a natural polymorphism in non-B HIV-1 subtypes, specifically subtype C and CRF02_AG (83%) but appears at only a 6% occurrence in subtype B ART naïve patients (495). In contrast, M36 has this accessory/secondary resistance effect to PIs in HIV-1 subtype B (496).

Primary DRM, V82A/F/T/S selected by IDV and LPV does appear more frequently in subtype F1 than subtype B HIV-1 infected patients (497). In contrast, D30N results in highly impaired viral replication in HIV-1 subtype C whereas this D30N is a primary PI resistant mutation in HIV-1 subtype B infected patients receiving a PI-based treatment (498). Lack of DRM D30N in some subtypes may relate to lack of natural polymorphisms, L63P and V77I in subtype B compared to non-B subtypes (495) necessary to compensate for D30N fitness loss. Mutation/natural polymorphism L63P has been shown to compensate loss in viral fitness by the primary PI resistant mutation, L90M mutation (499) found at lower frequency among subtypes C, F1, and intersubtype BF compared to subtype B (500–502). Finally, emergence of M89T mutation which causes phenotypic resistance to ATV, NFV (nelfinavir) and LPV is influenced by presence of polymorphism M89 in subtypes A, C, and CRF01_AE, and L89 in subtype B viruses (503). The mutation L89M increases genetic barrier for emergence of L90M and reduces susceptibility to IDV, RTV and NFV in subtype F though remains susceptible in subtype B viruses with exception

of IDV (504). *In-vitro* assays also reveal possible polymorphisms associate with hyper susceptibility to PIs. For example, CRF02_AG viruses carrying protease polymorphisms G17E and I64M confer hypersusceptibility to NFV, ATZ, and IDV inhibition (505). Accessory mutation/polymorphism I93L is also associated with hypersusceptibility to LPV in subtype C viruses compared to HIV-1 subtype B viruses (506).

Subtype-specific resistance mutations to NRTIs and NNRTIs: Generally, amino acid substitutions conferring resistance to NRTIs in subtype B match those of non-subtype B (507). However, there is more rapid emergence of the K65R in RT coding region in patients infected with subtype C compared to individuals infected with subtype B, G, and CRF02_AG (508). Lysine 65 of HIV-1 RT confers resistance to TDF, ABC, didanosine (DDI), and d4T and is coded by AAG in subtype C and AAA in subtype B such that a single versus double transition G to A mutation (respectively) is necessary for the Arginine. Contrary, K65R is less frequent in subtype A compared to subtype B and C HIV-1 in patients on ART (509). Otherwise, the frequency of thymidine analog drug resistance mutations (D67N, K70R, L210W, T215F/Y) is similar in patients infected with HIV-1 subtype A, B and C and receive a thymidine analog (D4T or AZT) in a treatment regimen (509), though M41L has been found to be more frequent in subtype B than C infected patients (497).

In relation to NNRTI resistance, mutations in HIV-1 pol associated with NVP and EFV, K103N, V106M and, A98S occur more frequently in those infected with HIV-1 subtype C compared to subtype B. This was observed in both ART naïve and experienced patients receiving an NNRTI-based treatment regimen (510). Mutation K103N associated with NVP, and EFV, is less frequent in subtype A and D compared to HIV-1 subtype C (511), and Y181C mutation which reduces susceptibility to NVP, EFV, etravirine, and rilpivirine shows more prevalence in subtype B and C compared to subtype A (509). Minority species of K103N or Y181C detected by ultrasensitive PCR were found in 42% of subtype A/AE compared to 70-87% of subtype C HIV-1 infections (512). V106M is preferentially selected in subtype C under NVP/EFV therapy compared to V106A commonly seen in subtype B viruses (399,513). N348I mutation in connection domain of HIV-1 RT reduces susceptibility of NVP, EFV, AZT, and DDI and is commonly associated with M184V and TAMs in HIV-1 infected patients (514–517). There seem to be decreased susceptibility for EFV and NVP in subtype A and D viruses than for subtype B and C when N348I mutation is present (518). Rilpivirine is a second generation NNRTIs with a high genetic barrier to resistance

in vitro (519) but with reduced susceptibility in presence of E138A/K/Q mutations (520). In HIV-1 ART naïve and experienced patients, E138A/K/Q mutations are found at a higher frequency in HIV-1 subtype C than subtype B infected patients (521).

Chapter 2

2 HIV-1 treatment, drug resistance and subtypes in Uganda

2.1 Preface

Uganda is one of the countries which has overcome a historical high prevalence of HIV-1 infection from 30% in 1992 when 18% of population was infected to the current 5.7% reported in 2018 (522,523). Uganda was also one of the first countries in Africa to access/distribute cART and with Dr. Anthony Fauci, was the primary architect of the U.S. President's Emergency Plan for AIDS Relief (PEPFAR) treatment plan. Despite the success of NNRTI-based first-line therapy under PEPFAR and UNAIDS/Global Fund in Uganda, there has been increasing prevalence of HIVDR leading to the implementation of PI-based second-line and RAL-based third-line treatment regimens (always with two NRTIs). Uganda has recently surpassed the WHO threshold of >10% prevalence of pretreatment HIV-1 drug resistance (PDR) to NNRTIs (524). WHO recommends a change of first-line regimens with PDR prevalence > 10% and Uganda has switched from NNRTIs+2NRTI for first-line to DTG/3TC/TDF regimen for patients initiating cART (525). DTG not only prevents the rapid emergence of resistance with first line cART but is also effective in viruses harboring most DRMs. Uganda is among few countries with a very diverse population of HIV-1 strains which makes studying HIV-1 subtype dynamics possible. Indeed, HIV-1 subtypes have been shown to influence rate of replication, pathogenesis, and occurrence of HIVDR in some HIV-1 infected populations. In this chapter, we describe ART options, HIVDR, and role of HIV-1 subtype distribution on treatment outcomes in the Uganda population.

2.2 HIV-1 history in Uganda

For many years, HIV-1 spread in Uganda without knowledge of what exactly was causing the “slim” body stature in individuals who were immunocompromised. By the time HIV-1 was recognized as a causative agent for AIDS, the rate of HIV-1 prevalence had already skyrocketed with over 18% of general population harboring HIV-1. HIV-1 was first recognized in Rakai district in South west part of Uganda in 1982 by a young resident doctor who was seeing 29 patients having clinical manifestations including fevers and sweats, oral candidiasis, diarrhea, Kaposi’s

sarcoma, lymphadenopathy, and genital warts. All these patients had uniform characteristic of extreme weight loss which was locally referred to as “slim” (3,526). In 1986, 900 cases were reported in the country (Uganda AIDS control program, 1989). Despite the first HIV-1 cases being described in 1982, HIV-1 epidemic in Uganda likely originated in 1960 (1950-1968) for subtype A_1 and 1973 (1970-1977) for subtype D in regions of Masaka (South-West of country) and then spread to the Kampala capital region (527). The 1970s-1980s was likely the period of exponential growth and by 1985, HIV-1 prevalence was reported to be 11% among population of pregnant women (528) and 18% in the general population. By 1992, HIV-1 prevalence in pregnant women receiving antenatal care had risen to 30% and 3% in urban and rural areas, respectively. Through public sensitization and behavioral changes including reduction of casual sex patterns and use of condoms, there was a sharp decrease in HIV-1 prevalence among pregnant women from 30% in 1992, to 21% in 1998 and 6% in 2000 (523). The rate of HIV-1 in adults has been steady over the last few years with prevalence of 7% in 2001, 6.5% in 2009, and most recently 6.8% in 2019 (529,530).

2.3 ART treatment in Uganda

The government of Uganda in collaboration with UNAIDS launched a two-year pilot program, Drug Access Initiative (DAI) in 1997 with mandate of improving HIV-1/AIDS care access for individuals living with HIV-1. The initiation of DAI program allowed over 912 HIV-1 infected individuals to access ART through the program (531,532). However, the extremely high annual cost of HIV-1 drugs to the tune of US $ 12,000 in 1997 to about US $7,200 in 1999 for first-line regimen was a huge stumbling block to the program (531). The ART program in Uganda only changed trends of HIV-1 infections after importation of generic drugs by the Joint Clinical Research Centre (JCRC) in 2000 which allowed patients to access ART at an end-user price of US $ 31/month for generic ARVs (AZT/3TC/NVP) produced by Cipla in India breaking the inequitable international TRIPS agreement (531,532). This allowed more patients access ART with some economic means. Despite the multinational pharmaceutical companies signing an agreement to lower the cost of ART for developing countries in May 2000, the most significant reductions in cost of ART coincided with importation of generic drugs from Cipla by JCRC in October 2000. Indeed, the number of HIV infected patients with access to generic ART at JCRC

increased from 912 in 2000 to 3,400 in 2002 (532). Further progress was made with initiation of PEPFAR program in 2004. By 2005, 75% of HIV-1 infected patients were receiving ART in Uganda (533). JCRC being one of major PEPFAR grantee, 19,000 HIV-1 infected patients were receiving ART in the first 18 months of PEPFAR including 2,000 orphans and pregnant women (533). JCRC kept playing a critical role with over 60,000 HIV-1 infected patients receiving treatment through the JCRC by the mid-2000s and nearly 200,000 through clinics across the country. In 2017, the Uganda government adopted 2015 WHO recommendation of "test and treat" which entailed initiating ART for all individuals testing HIV-1 positive, irrespective of their CD4 count or clinical stage.

First-line regimens in Uganda: In 1997-2000, cART consisted of three affordable choices for patients with high personal incomes: 2 NRTIs + either NNRTI or PI; or 2NRTIs + hydroxyurea (the latter showing only partial suppression and never used widespread in high income countries). In 2002, treatment guidelines recommended use of triple therapy of AZT or D4T plus 3TC/NVP as first line in Uganda despite limited access to this treatment for vast majority of Ugandans (534). The revised ART guidelines of 2008 recommended first line of AZT/3TC/NVP or EFV in both adults and children/infants but EFV was not recommended in children <3 years and <13 kgs (526). By 2008, if an infected individual had access to a clinic and had CD4 cell counts <200/mm3, this treatment was available for free through the PEPFAR, the Global Fund, or the Clinton Foundation. Since first roll-out of ART in Uganda in 2002, ART regimen constituted NNRTIs+ NRTIs with a number of transitions including d4T replacing AZT in 2008 and TDF replacing d4T (or AZT if still in use) by 2012. D4T and AZT had long since been abandoned by the high income countries due to mitochondrial toxicity, anemia and peripheral neuropathy (526,531,535). Due to favourable properties of DTG including high potency, low toxicity, higher genetic barrier to resistance, and increased circulation of NNRTI resistance, there was a switch in the cART naïve from an NNRTI-based treatment to the TDF/3TC/DTG combination (525). In March 2018, Uganda transitioned from NNRTIs-based to once-daily fixed dose of TDF/3TC/DTG for all patients initiating ART and eligible ART experienced patients except women and adolescents of childbearing potential or on effective contraception (525). In children 3<10 years and less than 35 kg ABC/3TC/LPVr is recommended (525). The updated 2020 HIV-1 treatment guidelines now recommend initiation of TDF/3TC/DTG in all adults and adolescents ≥ 30kg including pregnant and breastfeeding women with use of EFV (400mg) where DTG is contraindicated (536). In children ≥ 20Kg - <30Kg,

ABC/3TC/DTG is recommended first-line with LPV/r, EFV, RAL, or triple NRTIs use when DTG is contraindicated (536). A study at one of the HIV-1 treatment specialized centers in Uganda, shows possible increased prevalence of hyperglycemia in patients who transitioned to DTG though this group had significantly higher number of older, male, and long-term ART experienced patients compared to patients who did not transition to DTG (537).

Second-line regimens in Uganda: ART expansion phase starting in 2002, recommended a second-line of AZT or d4T plus DDI/LPV/r (534). The revised cART treatment and care guidelines of 2008 recommended ABC/DDI/LPVr, FTC/LPVr, or AZT(or ABC)/DDI/LPVr as second-line regimen in adults and children/infants respectively (526). In 2018 national HIV-1 treatment guidelines recommended second-line of AZT/3TC/ATVr in adults and AZT/3TC/RAL or DTG in children 3<10 years (525). The 2020 HIV-1 treatment guidelines recommend TDF/3TC/LPV/r second-line in ≥ 30kg patients failing on DTG-based first-line, TAF or ABC/3TC/LPV/r in children ≥ 20Kg - <30Kg, and ABC/3TC/LPV/r in children weighing <20Kg (536).

Third-line regimens in Uganda: Viral load of <1000 copies/ml with evidence of >95% adherence to treatment warrants a switch to third-line regimen under guidance of genotyping test (525). The choice of third-line regimen is guided by HIV-1 genotype testing, and regimens are selected by national third-line ART team basing on genotypic profile, available ART options and patient treatment history (525). Third-line treatment consists of regimens active against resistant viruses and commonly DRV/r, RAL, and ETR, or two recycled NRTIs are used in these highly treatment experienced patients. Third-line cART remains the last line of treatment due to inaccessible CCR5 or fusion inhibitors in Uganda. Ugandan children (<18 years) carrying multiple DRMs after failure on second-line, responded well to RAL-based third-line after ~5 years of follow up (538).

2.4 Distribution of HIV-1 subtypes in Uganda

HIV-1 subtypes A and D account for the majority of HIV-1 infections in Uganda (539,540), with subtype C (541–543), B (542,544,545), and G (546) only rarely reported in the country. The A/D unique and stable recombinant forms (URFs and CRFs) account for 9% to 30% of HIV-1 infections (540,547,548). Subtype C and other CRFs account to <10% of HIV-1 infections in Uganda (540).

HIV-1 subtype A emerged in Uganda earlier than subtype D (549) and it was shown to have originated from Congo which then spread from East to West in the country with subtype A now predominant in HIV-1 infections of Kenya. HIV-1 Subtype D in Uganda is said to have originated from Tanzania but originated in the Congo Basin like all HIV-1 group M strains. Subtype D now predominates the Southwest of the country and has had more limited spread to the North and East (550–552). Consequently, distribution of HIV-1 subtypes vary in different geographical locations in the country. In central regions of the country-areas of Kampala and Entebbe, subtype A is only slightly more prevalent than D (50 and 30% respectively) (539,548,553) with subtype A becoming more prevalent near the Kenyan border; and subtype D reaching a peak in prevalence (>70%) in the Southern Rakai district (550,554). Despite HIV-1 subtype D being of the highly prevalent HIV-1 strains, its prevalence has been steadily decreasing while subtypes A, C, and A/D and A/C URFs have been increasing in Uganda (555). HIV-1 subtype A is easily spread through personal and community transmission (556), however, with increased access to more potent cART, the incidence rates of subtype A will also likely decrease in the population. By using phylogenetic analyses, HIV-1 transmission dynamics through clusters has been studied through a cohort of HIV-1 patients in Southwestern Uganda. In a 7-year (2004-2010) study of 94 participants, 37% of sequences (partial *gag* and *env*) accounted to 13 transmission clusters and revealed a possible high sexual risk behavior and super transmitters in the community (552). Phylogenetic analysis of 3796 HIV-1 pol sequences from HIV-1 infected patients in Southwestern Uganda, identified 524 clusters and clustering was largely associated with being a female and staying within general population cohort set up previously to analyze trends in HIV-1 prevalence and incidence, their determinants and pathogenicity. The clustering dynamics revealed significant viral spread from general population cohort without viral introduction from outside the cohort (557).

2.5 Role of HIV-1 subtype on body response to infections

In Uganda, HIV-1 subtype is associated with different clinical and disease manifestations. HIV-1 subtype D is associated with faster disease progression than subtype A in a Uganda population (558). In ART naïve and experienced HIV-1 infected patients, cART failure is more frequent in subtype D compared to subtype A infected individuals (488). Subtype D infected is also associated with lower $CD4^+$ T cell count and faster progression to death and AIDS, compared to subtype A

(539,558). Subtype D also shows significantly lower transmission rate than subtype A among HIV-1 discordant couples (556). Increased ability to use both CXCR4 and CCR5 coreceptors during diseases progression with subtype D (559), contrary to an almost exclusive use of CCR5 by HIV-1 subtype A (558,560) may perhaps explain the high virulence of subtype D. HIV-1 infected Ugandans infected with CCR5 viruses have higher CD4 cell count than CXCR4 or CCR5/CXCR4 viruses (561). However, independent of co-receptor usage, HIV-1 subtype D isolates have higher replicative fitness in primary human T cells when compared with HIV-1 subtype A isolates and higher replicative fitness of subtype D HIV-1 directly correlated to the faster rate of disease progression as measured by a sharper decline of CD4 T cells in blood (562). This faster decline CD4 T cells was observed without any usage of CXCR4 by subtype D HIV-1.

Subtypes A and D infected Ugandans also have increased $CD4^+$ and $CD8^+$ T cell activation compared to subtype B infected counterparts (563). CD4 dysfunction characterized by higher expression of programmed cell death receptor -1 (PD-1) and increased cell apoptosis is more pronounced with subtype D compared to subtype A in ART naive Ugandans (564). Subtype D infected patients also exhibit pronounced loss of invariant natural killer T (iNKT) cells whereas FoxP3+ regulatory T cells (Tregs) loss is more frequent in subtype A infected Ugandans (565). On contrary, there seem to be no difference in the initial cART response among patients infected with HIV-1 subtype A, C and D (566).

2.6 HIV-1 drug resistance in Uganda

In Uganda, the high prevalence rates of HIV-1 infections (~5.7%) (567) and frequent use of low genetic barrier to resistance regimens have led to increased levels of HIVDR. Uganda much more like the rest of Africa countries, first generation NNRTIs, NVP and EFV have constituted first-line combination (531,568) and difficult to take LPV has frequently been used in second-line treatment (526). The high prevalence of HIVDR can also be explained by early roll out of ART in 2002 compared to other African countries (534). Overall, over 10% of HIV-1 infected Ugandans on ART harbor drug resistance variants, and over 15.4% initiate ART already carrying HIV-1 transmitted drug resistance (TDR) (524).

HIVDR in ART naïve patients and those with previous exposure to ART has been increasing in Uganda (569). Over the years, the frequencies of HIVDR in patients initiating ART in Uganda has increased from 0% (2006–2007) (570), to 8.6% (2009–2010) (571) and 15.4% from WHO survey (2014–2016) (524). This is largely contributed by NNRTIs-associated TDR compared to other classes of ART. HIV-1 infected patients carrying TDR can achieve viral suppression on first-line (572), however, escalating TDR beyond 10% threshold, called for substitution of NNRTI component following WHO treatment guidelines (524). In ART naïve children and > 12 years, HIVDR to first-line between 2010-2011 was found at 7.7% (573), and 16.9% (574). Among different patient groups, TDR among fisher folks has been observed between 5-15% for NNRTIs and <5% for NRTI and PI regimens (575), 3% for NNRTIs among female sex workers (576), and <5% for NRTI, NNRTI and PR among women attending antenatal care (570).

HIVDR to thymidine (AZT, d4T) and non-thymidine analogues used in first-line regimens has been observed in 58.8-95% HIV-1 infected Ugandans (540,577). Thymidine associated mutations, M41L, D67N/G, T215I/F/Y/T, and K219N/K/E/Q, are frequently selected in HIV-1 infected Ugandans failing on first-line ART (540,577,578). Non-TAM, M184V/I, K65R/K, are the most prevalent mutation observed in patients failing first-line regimens (540,577,579,580). NNRTIs associated resistance account to the majority of first-line resistance with 73.2-96% of first-line failures harboring NNRTIs-associated resistance (540,577,581). The major frequently selected NNRTIs mutations include, K101E/Q, K103N/S, G190A/S/E/G, and Y181C (540,577–579,582). NNRTIs major resistance mutations, K103N, G190A, and Y181C have low fitness cost and therefore frequently observed even after many years after switching to non-NNRTI regimen (583,584). We have shown previously that over 55% of patients failing PI-based second-line in Uganda harbor NNRTIs- resistance mutations (540). There has also been a challenge of using NVP monotherapy to prevent mother to child transmission which may predispose women to HIVDR. Indeed the risk of HIV-1 resistance after a single-dose NVP has been estimated to be 32% among Ugandan women (585,586). Starting babies with prior exposure to single dose NVP to NNRTIs (due to lack of pediatric PI formulations) further exposes them to NNRTIs resistance. HIVDR has been observed in over 35.7% of children in prevent mother to child transmission program in Uganda (573). In some cases, children failing first-line and some with pre-exposure to single dose NVP, all have been found to harbour NRTI and NNRTIs resistance (587) and impact of HIVDR in children < 3 years is stronger and has been associated with VF and acquired drug resistance

(574). Children failing first-line and harboring common M184V, K65R/N, and K103N mutations may respond well on boosted LPV containing second-line regimens (588). At first-line failure, CD4 cell count at baseline <250 cells/ul and viral load > 100,000 copies/ml have been shown as independent predictors of HIVDR at 12 months in HIV-1 infected Ugandans (554,577). The type of NRTI+ NNRTIs-first-line regimen has been associated with accumulation of TAMS and K103N, and poor adherence predicts emergence of K103N and reduced virological response to PI-based regimens in Ugandan children (588). Starting HIV-1 patients on AZT containing first-line compared to TDF, and ART duration of >82 weeks have been shown to be independent predictors of acquired HIVDR (589).

Following two consecutive viral load of <1000 copies/ml with 3-6 months apart and adherence support after first viral load test, patients failing on first-line are switched to PI-based second-line, regimens (525). The boosted LPV has been commonly used in second-line combination until recently when the country switched to boosted ATV and 2 NRTIs (525). PIs have higher genetic barrier to resistance compared to NNRTIs or NRTIs. In patients failing PI-based second-line, PI resistance has not or nearly observed (578,581) but also detected at 18.5% (590), and 19.4% (579) in Ugandan patients. Major PIs mutations, V82A, I54A, M46I/L are frequently observed in patients failing PIs-based second line in the Uganda (540,579,590). In patients failing second-line and carrying PI-resistance, DRV remains the only active part of PI cocktail for third-line treatment (579). PI resistance at first-line failure is very low in children (<12 years) and over 20% fail second-line but lacking PI resistance (580). PI resistance has also been shown to be rare in children (<20 years) failing second-line (588). Sub-optimal adherence and less weight have been associated with HIV-1 treatment failure of second-line in Ugandan children (<12 years) (580).

Data on HIVDR to third-line regimens in Uganda is very limited. In patients failing RAL-based third-line, over 48% of patients carry RAL/EVG associated primary DRMs (540). About 5% of patients failing RAL-based third-line regimens carry DTG-associated DRMs and 2-10% harbor secondary mutations against INSTIs. Among major DRMs against INSTIs, N155H mutation is most frequently selected and Q148R/K least selected INSTIs associated mutation in HIV-1 infected Ugandans failing third-line regimens (540). In INSTIs naïve HIV-1 patients, major INSTIs mutations, Y143H, and Q148R only are observed at low frequency (1.1-1.6%) (578). HIV-1 infected patients from 4 African countries including Uganda and initiated on RAL-intensified

first-line regimen, 9% were found with RAL/EVG resistance but no high-level DTG resistance (591). Primary INSTIs associated DRMs in ART naïve patients in Uganda and other 4 African countries have been detected only at <20% frequency (592).

Despite higher entropy scores or amino acid variability at sites where INSTIs mutations are selected for primary or secondary INSTI resistance between subtype B and non-B HIV-1 subtypes, there is very limited data on prevalence, genotype, and impact on susceptibility to INSTIs by these DRMs in non-B HIV-1 subtypes. Observations in the subsequent chapters show absence of INSTIs associated mutations in INSTIs naïve (n=366) but in 47% of patients failing on RAL-based regimen. Among the 47% with INSTIs DRMs, 4% (2/51) of patients (subtype A and D) had multiple primary INSTIs DRMs conferring resistance DTG, BIC, and CAB, a unique observation in both LMICs and HICs. The viruses harboring single RAL/EVG primary INSTIs mutations (~50%) remained susceptible to DTG, BIC, and CAB with fold-change in EC_{50} values nearly equivalent to that of wild-type. We confirmed that subtype A with E138A/G140A/G163R/Q148R and subtype D with E138K/G140A/S147G/Q148K viruses on RAL-based regimen are highly resistant to all currently available INSTIs in LMICs. Emergence of INSTIs resistance appears in part to be subtype specific with even N155H reducing DTG susceptibility more so in subtype A and D than in subtype B HIV-1. In contrast to commonly observed G140S/Q148H mutations in subtype B viruses, non-B HIV subtypes commonly carried G140A/Q148KR mutations. The potential novel resistance mutations to INSTIs, I203M and I208L, did not singly reduce susceptibility to either RAL or DTG, further analysis is warranted to determine how these novel mutations influence susceptibility to INSTIs in HIV-1 subtype A and D infected patients.

Chapter 3

3 Absence of HIV-1 drug resistance mutations supports the use of Dolutegravir in Uganda

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All of the experimentation on this study was performed and/or supervised by Emmanuel Ndashimye

3.1 Preface

Currently there are over 37 million people infected with HIV-1 worldwide and over 35 million people have died due to HIV-1 related complications. Since the introduction of the first ART regimen, AZT in 1987, there have been tremendous progress in different ART regimens to the tune of 40 different regimens available today. In the due course, more potent ART regimens with better tolerability, safety, and activity with resistant viruses have been made accessible. This achievement has to the great extent been realized in HICs which have capabilities to get access to these better, high-cost drugs. INSTIs are the latest class of ART in this category of drugs with high potency and yet good safety profile.

Dolutegravir, one of the second generation INSTIs, was approved in 2013 by FDA but Uganda much more like other LMICs could only have DTG widely accessible in late in 2018. DTG was introduced in Uganda in form of a triple combination of DTG/3TC/ABC in 2019. Yet still, data on DTG susceptibility in subtype A, D, C and recombinant viruses commonly circulating in Uganda is not available. The study presented herein, tacked this challenge by screening for DTG, RAL and EVG associated DRMs in HIV-1 infected individuals on different lines of ART in Uganda. I selected this cohort of patients, carried out laboratory assays, and completed the statistical analyses for the study.

3.2 Abstract

Objectives: To screen for drug resistance and possible treatment with Dolutegravir (DTG) in treatment naïve patients and those experiencing virologic failure during first, second, and third line combined antiretroviral therapy (cART) in Uganda.

Design: Samples from 417 patients in Uganda were analyzed for predicted drug resistance upon failing a first (N=158), second (N=121), or third line (all 51 involving Raltegravir [RAL]) treatment regimen.

Method: HIV-1 pol gene was amplified and sequenced from plasma samples. Drug susceptibility was interpreted using the Stanford HIV-1 database algorithm and SCUEAL was used for HIV-1 subtyping.

Results: Frequency of resistance to nucleoside reverse transcriptase inhibitors (NRTI) (95%) and non-NRTI (96%) was high in first-line treatment failures. Despite lack of NNRTI-based treatment for years, NNRTI resistance remained stable in 55% of patients failing second line or third line treatment and was also at 10% in treatment naïve Ugandans. DTG resistance (*n*=366) was not observed in treatment naïve or individuals failing first and second line cART and only found in two patients failing third line cART while 47% of the latter had RAL and Elvitegravir (EVG) resistant HIV-1. Secondary mutations associated with DTG resistance were found in 2% to 10% of patients failing third line cART.

Conclusion: Of fourteen drugs currently available for cART in Uganda, resistance was readily observed to all antiretroviral drugs (except for DTG) in Ugandan patients failing first-, second-, or even third-line treatment regimens. The high NNRTI resistance in first line treatment in Uganda even among treatment naïve patients, calls for the use of DTG to reach the UNAIDS 90:90:90 goals.

3.3 Introduction

By 2016, there were 36.7 million people living with HIV-1 infection worldwide, and 1.8 million new infections diagnosed in the same year (593). Uganda is among the countries with the highest burden of HIV-1 infections, with approximately 1.5 million (7.1%) people living with HIV-1/AIDS and 57% of them receiving combined antiretroviral therapy (cART)(594). Unfortunately, due to various sociological and economic factors, HIV-1 drug resistance has been rapidly emerging in patients receiving cART such that HIV-1 drug resistant variants are now responsible for at least 9% of the new infections(488).

In Uganda, as most of sub-Saharan Africa, over 57% of patients have access to TDF- and EFV-based first line regimen recommended by the World Health Organization (WHO), while less than 50% of patients are receiving the DTG- and DRV-based first-line regimens recommended by the International Antiviral Society – USA(595). However, rapid emergence of HIV-1 drug resistance to first line B1a rated treatment regimens argues for increasing access to more effective cART, which may lead to better treatment outcomes, lower adverse events/drug toxicity, and higher barriers for drug resistance. DTG, a second-generation Integrase Strand Transfer Inhibitor (INSTI), Elvitegravir/cobicistat (EVG/cobi) or Raltegravir (RAL) are three "backbone" INSTIs recommended for first line treatment regimens in combination with tenofovir disoproxil fumarate/emtricitabine (FTC) (or with tenofovir alafenamide/FTC when combined with EVG/cobi)(596). DTG/abacavir/lamivudine is also employed if the patients are screened to exclude those with an HLA B57 allele. Rilpivirine along with the latter NRTIs are used for new first line treatment regimens. Despite these preferences for treatment in HICs, at the JCRC in Uganda, currently less than 1% of the 60,000+ patients receive this first line cART recommended by the CDC for HICs.

Equally important, *in vivo* DTG resistance has rarely been observed even in those few patients showing virological failure in NRTI-experienced but INSTIs-naïve patients(435). *In vitro* studies characterizing the DTG-associated R263K mutation in subtype B, C, and CRF02_AG HIV-1 strains, showed early emergence of R263K in subtypes B and CRF02_AG, whereas the G118R substitution was only observed in subtype C and CRF02_AG (429). It is possible that HIV-1 strains from different subtypes may explore distinct paths, selecting for different mutations, to escape DTG pressure. Moreover, while resistance to EVG and RAL comes at high fitness cost

(597), the DTG-resistant R263K mutation is even slower to emerge and more debilitating to HIV-1 replication. The R263K mutation observed in clinical and *in vitro* studies, and secondary mutations such as H51Y, M50I and E138K observed in cell culture studies, do emerge during DTG treatment but appear unable to restore replication capacity of the virus (434,598,599). DTG can also inhibit most HIV-1 isolates resistant to RAL and EVG which relates to its success in RAL-experienced patients in the VIKING-3 study (318).

Despite the strong safety profile and success of DTG in both first and second line treatments, its use in LMICs has been minimal. DTG has been extensively tested in patients infected with subtype B, with no difference on DTG susceptibility been observed in non-B subtype HIV-1 strains in dose-escalating/selection experiments (600). However, little is known about DTG treatment outcomes in patients infected with subtype A and D HIV-1 primarily found in Uganda (601).

In this study, we evaluated the possible treatment with DTG in cART to treat HIV-1 infected Ugandan individuals based on the current drug resistance profile in treatment naïve and highly treatment experience patients. Drug susceptibility was predicted in 417 plasma samples from treatment naïve individuals (N) (n=87), first-line failures (FF) (n=158), second-line failures (SF) (n=121) or third line/RAL-based antiretroviral failures (RF) (n=51). Our results did not predict DTG resistance in HIV-1 infected treatment-naïve patients, nor from individuals failing different cART regimens, highlighting the suitability of DTG-based therapies to treat HIV-1 infected patients in Uganda at any stage of the disease.

3.4 Materials and methods

Samples for the study

This was a retrospective study that assessed the prevalence and impact of DTG associated mutations in patients failing on different treatment regimens. Samples were collected from the WHO, CAP, and NIH-VQA-accredited Center For AIDS Research (CFAR) Laboratory of the Joint Clinical Research Center (JCRC) in Kampala, Uganda. The JCRC is one of the first HIV-1 treatment centers in the country to roll out cART and currently the only site licensed to provide INSTIs in the country. The patient database in the CFAR laboratory was used to access the patient demographics, medical, and treatment history of the study samples. A total of 440 plasma samples were collected from patients receiving routine treatment care at the JCRC and with virological failure, defined by a viral load above 1,000 copies/mL and/or $CD4^+$ T-cell counts below 250 cells/mm^3. These virological failures included plasma samples from 90 N, 165 FF, 125 SF, and 60 RF patients. Antiretroviral therapy (ART) naïve samples came from the Pan-African Studies to Evaluate Resistance (PASER) network (602), the Monitoring Antiretroviral Resistance in Children (MARCH) observational cohort study (603), and the Hormonal Contraception and HIV-1 Genital Shedding and Disease Progression among Women with Primary HIV-1 Infection (GS) study (604). Forty two of 165 FF samples came from the Europe-Africa Research Network for Evaluation of Second-line Therapy (EARNEST) trial (605). The rest of the samples were patient samples collected during routine Sanger genotyping testing at the JCRC. Ethical clearance was obtained from the IRBs at the JCRC and UHCMC/CWRU (EM-10-07 and 10-05-35)

RNA extraction and PCR amplification

Viral RNA was extracted from 440 plasma samples using a QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription of the full-length HIV-1 integrase (IN)-coding region from extracted viral RNA and amplification was done with the sense primer RTA9F (5′-TATGGGGAAAGACTCCTAAATTTA-3′) and antisense primer 3Vif (5′-AGCTAGTGTCCATTCATTG-3′) using a Superscript III single RT-PCR system with Platinum Taq DNA polymerase kit (Thermo Fisher Scientific) as per manufacturer's instructions. Nested PCR was done using the sense primer INTFEXT1 (5′-AGAAGTAAACATAGTAACAGACTCACA-3′) and antisense Vif 3 reverse 1 primer (5′-GTCCTGCTTGATATTCACACC -3′) using a Platinum Taq kit (Thermo Fisher Scientific) as per manufacturer's instructions to generate amplicon of 1433 base pairs. Amplification of protease (PR) and reverse transcriptase (RT) regions was done as previously described (488). The amplicon was purified using ExoSAP-IT enzyme (Thermo Fisher Scientific) and quantified using a Qubit fluorometer (Thermo Fisher Scientific).

Cycle sequencing and sequence analysis

The HIV-1 IN as well as the PR and RT coding regions were amplified and analyzed on a Sanger sequencing platform. Briefly, quantified and purified PCR product was sequenced with primers spanning the full length of the IN gene (1-288 amino acids): Vif 3 reverse1 (5′-GTCCTGCTTGATATTCACACC-3′), INTREXT (5′-AATCCTCATCCTGTCTAC -3′), and INTFEXT1 (5′-AGAAGTAAACATAGTAACAGACTCACA-3′). PCR product was sequenced with ABI Big dye terminator (v3.1) (Thermo Fisher Scientific) according to manufacturer's instructions on ABI 3730xl sequencing platform (Life Technologies, Carlsbad, California, USA). A total of 417 samples (N = 87, FF = 158, SF = 121, and RF= 51) were amplified and sequenced successfully for the IN gene (Fig. 17, Table 2).

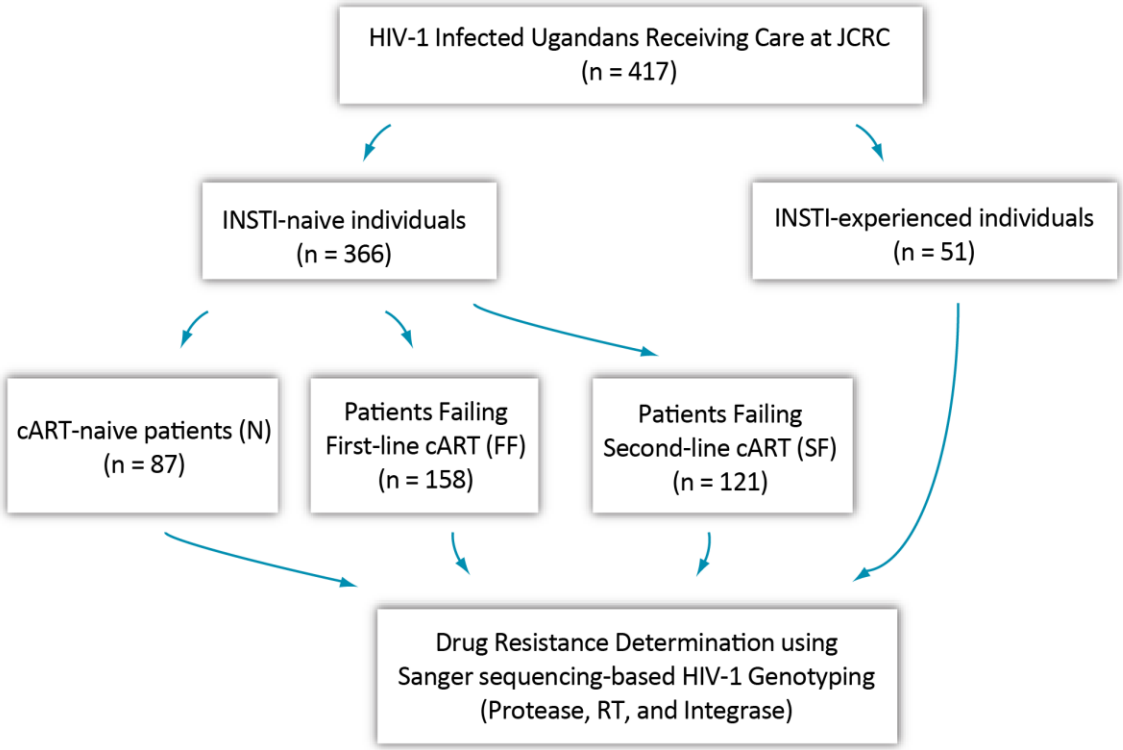

Figure 17. The workflow chart of the patient numbers and their respective groups. FF consisted of patients who were on NNRTI-based combination therapy, SF had PI-based combination, RF had RAL backbone and N had not been exposed to ART. Abbreviations: INSTIs, Integrase Strand Transfer Inhibitors, RT, reverse transcriptase, PR, protease.

Sequences were analyzed using RECall (beta v3.02) program as recommended by the WHO(606).

Table 2: Clinical and virological characteristics of the patients in the study

			Subtype No. (%)				
Group of patients [a]	cART regimen (No. patients)[b]	Mean HIV-1 RNA log^{10} c/ml [c]	A	D	C	A/D	Other [d]
cART naïve (n = 87)	None	4.64	42(47.1)	17(19.5)	3(3.4)	9(10.3)	17(19.5)
Failing First-line cART (n = 158)	AZT, 3TC, NVP (38)	0.54	14(36.9)	11(28.9)	4(10.5)	2(5.3)	7(18.4)
	TDF, 3TC, EFV (34)	1.21	14(41.2)	10(29.4)	2(5.9)	1(2.9)	7(20.6)
	AZT, 3TC, EFV (18)	1.31	18(44.4)	6(33.3)	1(5.6)	1(5.6)	2(11.1)
	TDF, 3TC, FTC (15)	0.43	1(20)	1(20)	1(20)	0(0.0)	2(40)
	Other (14)	7.5	5(35.7)	5(35.7)	2(14.3)	2(14.3)	0(0.0)
	3TC, D4T, NVP (13)	2.2	8(61.5)	1(7.7)	0(0.0)	1(7.7)	3(23.1)
	ABC, 3TC, NVP (10)	2.56	4(40)	1(10)	1(10)	1(10)	3(30)
	ABC, 3TC, EFV (7)	0.64	7(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	TDF, 3TC, NVP (6)	0.97	0(0.0)	3(50)	0(0.0)	1(16.7)	2(33.3)
	3TC, AZT, NVP (4)	2.22	0(0.0)	1(25)	1(25)	1(25)	1(25)
	d4T, 3TC, NVP (3)	0.97	0(0.0)	3(100)	0(0.0)	0(0.0)	0(0.0)
	AZT, 3TC, ABC (2)	ND	0(0.0)	1(50)	0(0.0)	0(0.0)	1(50)
	FTC, TDF, EFV (2)	0.7	1(50)	0(0.0)	0(0.0)	0(0.0)	1(50)
	TDF, ABC, AZT (n = 2)	ND	1(50)	0(0.0)	0(0.0)	0(0.0)	1(50)
Failing Second-line cART (n = 121)	TDF, 3TC, LPVr (30)	1.22	13(43.3)	4(13.3)	2(6.7)	2(6.7)	9(30)
	TDF, 3TC, ATVr (30)	2.2	14(46.7)	7(23.3)	1(3.3)	2(6.7)	6(20)
	ABC, 3TC, LPVr (25)	2.13	12(48)	8(32)	1(4)	0(0.0)	4(16)
	ABC, 3TC, ATVr (25)	3.64	3(60)	0(0.0)	0(0.0)	0(0.0)	2(40)
	AZT, 3TC, LPVr (13)	7.82	4(30.7)	3(23.1)	0(0.0)	3(23.1)	3(23.1)

	LPVr (7)	0.05	3(42.9)	4(57.1)	0(0.0)	0(0.0)	0(0.0)
	AZT, 3TC, ATVr (6)	2.53	4(66.7)	2(33.3)	0(0.0)	0(0.0)	0(0.0)
	Other (5)	0.7	1(20)	1(20)	0(0.0)	1(20)	2(40)
Failing RAL-based cART (n =34)	RAL, LPVr (16)	2.2	7(43.75)	4(25)	0(0.0)	3(18.75)	2(12.5)
	Other (6)	0.3	4(66.6)	1(16.7)	0(0.0)	0(0.0)	1(16.7)
	RAL, DRVr (5)	9.5	4(80)	1(20)	0(0.0)	0(0.0)	0(0.0)
	RAL, ATVr (2)	8.5	1(50)	1(50)	0(0.0)	0(0.0)	0(0.0)
	RAL, TDF, 3TC, LPVr (2)	2.7	2(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	TDF, 3TC, RAL, DRVr (2)	0.03	1(50)	1(50)	0(0.0)	0(0.0)	0(0.0)
	RAL, ETR, DRVr (2)	1.1	2(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)

The HIV-1 subtype was predicted using SCUEAL subtype classification algorithm. Viral loads were assayed using Abbott m2000sp/rt or Roche COBAS Amplicor Monitor ultrasensitive tests, v1.5. [a]The total number of patients tested for INSTIs resistance in each patient group. [b]Description of the ART combinations in the study, and the number of patients taking that combination. ND, not determined. Other, the number of ART combinations which were less prescribed i.e., only one patient for each of these ART drug combinations: (3TC, TDF, NVP) (AZT, D4T, 3TC, NVP) (3TC, EFV, LPVr) (3TC, EFV, ATVr) (ABC, DDI, LPVr) (TDF, 3TC, ATVr) (EFV, DDI, LPVr) (EFV, RAL, DRVr) (TDF,3TC, RAL, ETR) (TDF, FTC, RAL, DRVr) (RAL, ETR, LPVr). [c]The average number of patient viral loads (copies/ml x 10^5), ND, not determined. [d]The percentage of patients with HIV-1 unique circulating recombinant forms (CRFs); A1/C, A1/AE, D/U, J/A1,C/G, AE/D, A1/U, A3/U and CRF35. Until the time ATVr was available and incorporated into national treatment guidelines, LPVr was infrequently provided as second line monotherapy if resistance patient had drug resistance to 3TC and AZT.

Stanford resistance predictions were obtained using a Python client, SierraPy 0.1.2, to automate transactions with the Stanford HIV-1db Sierra web service algorithm v8.3 (607).

Subtype classification

The resulting JSON files were converted into CSV files using an in-house R script. SCUEAL was used for HIV-1 subtype classification and recombination detection (608). This algorithm maps sequences to a phylogeny of subtype reference sequences by maximum likelihood to classify subtypes and detect recombination. Sequences with two or more recombination breakpoints with multiple subtype or sub-subtype (e.g., A1, A2) parents are labeled "complex" recombinants. Amino acid polymorphisms were extracted by pairwise alignment of the consensus sequence to the HXB2 reference integrase gene sequence using an in-house Python script. Insertions relative to this reference were discarded and the aligned sequences were translated into amino acids with

a HXB2 coordinate system. We excluded circulating recombinant forms (CRFs) from the phylogenetic analysis and aligned all the data using MAFFT v7.305b(609), including the HIV-1 subtype reference sequences of HIV-1 subtypes A1, A2, C and D. We manually adjusted the resulting alignment using AliView v1.19-beta-3(610). A phylogenetic tree was reconstructed by maximum Likelihood using PHYML v20160207 with the default parametric bootstrap support analysis(611). The General Time Reversible model incorporating invariant sites and a gamma distribution for rate variation across sites (GTR+I+G) was selected using the Akaike Information Criterion (AIC) using jModeltest v2.1.10(612). The tree was visualized and manually annotated in FigTree (A. Rambaut, http://tree.bio.ed.ac.uk/software/ figtree) and Archaeopteryx v0.9920 beta(613).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.03 (GraphPad Software, La Jolla, CA) using one-way analysis of variance (ANOVA) unless otherwise specified.

3.5 Results

Based on sequencing of the PR, RT and IN-coding regions, HIV-1 subtype distributions did not significantly differ among the four groups (N, FF, SF, and RF). Subtype A virus was the predominant subtype found in nearly half of patients in each group followed by subtype D, and C. Our observations are consistent with our previous study on Uganda HIV-1 subtype distribution in the last 10 years (488) (Fig. 18). As previously described (488), a higher proportion of patients failing treatment appeared to be infected with subtype D, but this was not statistically significant (Fig. 18). No subtype C infections were identified in the RAL treated group, most likely due to the smaller sample size. AD recombinants were observed at higher frequency in the naïve than treatment failure populations whereas complex recombinants/CRFs comprised the remaining 13-19% (Fig. 18).

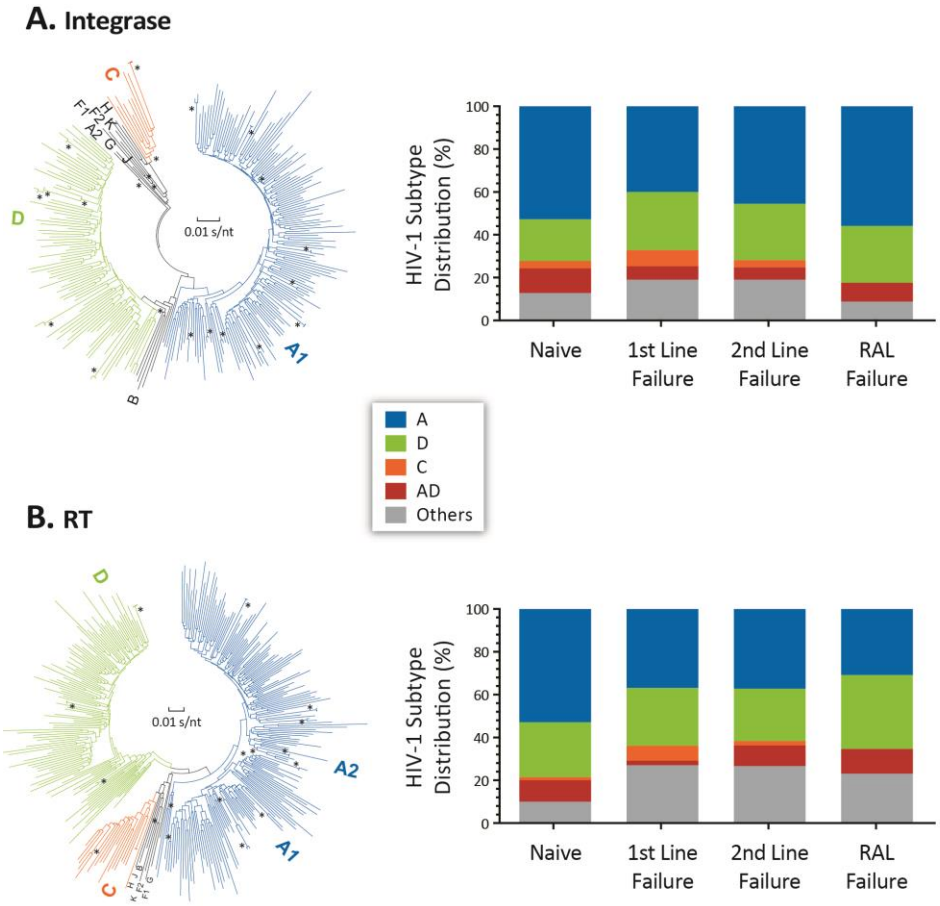

Figure 18. HIV-1 subtype classification of reverse transcriptase, protease, and Integrase regions. (A) The bar graph (Right) and phylogenetic tree (Left) describe the HIV-1 subtype classification of the IN gene (percentages) of ART naïve patients, FF, SF and RF. (B) The bar graph (Right) and phylogenetic tree (Left) of HIV-1 subtype classification of RT, and PR regions of N, FF, SF, and RF. Subtype descriptions are embedded in the figure. An in-house Python script was used to label tips with subtype classifications from SCUEAL. A maximum likelihood phylogenetic tree was reconstructed from the alignment using PHYML v20160207 with the default parametric bootstrap estimation of branch supports given the data. The tree was visualized and manually annotated in FigTree (available at: http://tree.bio.ed.ac.uk/software/figtree) and Archaeopteryx v0.9920 beta.

On average, 10% of the treatment naïve patients were infected with HIV-1 variants harboring primary resistance mutations to at least one NRTI or NNRTI (Fig. 19, Fig. 20). This treatment naïve group was recruited from 2007-2011 during chronic disease and tested for HIV-1 drug resistance genotype (602,603,605).

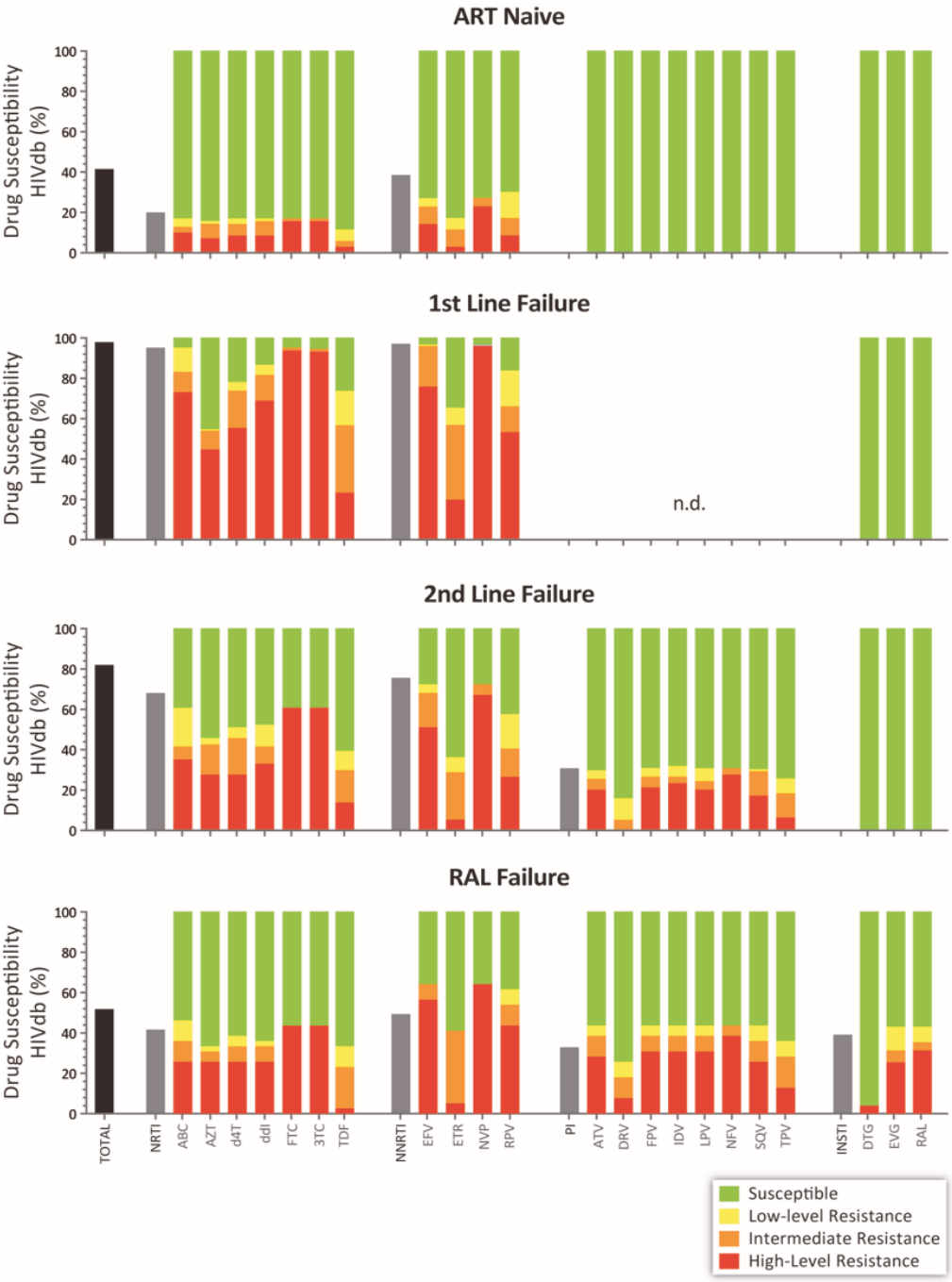

Figure 19.Drug resistance predictions based on pol sequences of treatment naïve, first, and second line treatment failures or in patients receiving raltegravir. Genotypic resistance/drug susceptibility prediction was performed on 400 HIV-1 pol sequences from Uganda using the HIV-1db genotypic resistance interpretation algorithm from Stanford University. Panels A, B, C, and D show the level of drug resistance in N, FF, SF, and RF respectively. The first bar in each patient group represents total drug resistance in that group, and the first bar in each drug class represent total drug resistance in that respective drug class. The rest of description of level of drug resistance is embedded in the figure.

A 10% incidence of drug resistance in chronically infected patients from 2007-2011 is likely an underestimate of current rates of transmitted drug resistance HIV-1 in the treatment naïve population. Recruitment of these patients during chronic disease likely resulted in a loss of drug resistance and reversion to wild type HIV-1 following initial infections with a drug resistant HIV-1 variant. A recent WHO report 2017 shows pretreatment HIV-1 drug resistance to EFV/NVP in 15.4% of the HIV-1 positive population in Uganda (524).

Figure 20. HIV-1 genotypic resistance interpretation based on Sanger sequencing. Amino acid substitutions was used with HIV-1db program Genotypic resistance interpretation algorithm from Stanford university HIV-1 drug resistance database (https://HIV-1db.stanford.edu) to predict the levels of susceptibility to PR, RT and INSTIs. A susceptible genotype is shown in green, intermediate and high-level resistance is shown in yellow and red respectively. Abbreviations: ABC (abacavir), AZT (zidovudine), D4T (stavudine), DDI (didanosine), FTC (emitricabine), 3TC (lamivudine), TDF(tenofovir), EFV(efavirenz), ETR (etravirine), NVP (nevirapine), RPV (rilpivirine), ATVr (atazanavir/r), DRVr (darunavir/r), FPVr (fosamprenavir/r), IDVr (indinavir/r), LPVr (lopinavir/r), NFV (nelfinavir), SQVr (saquinavir/r), TPVr (titranavir/r), DTG (dolutegravir), EVG (elvitravir), RAL (raltegravir).

A slightly higher frequency of NNRTI over NRTI resistance was observed in our treatment naïve population, which may be due to higher fitness costs of NRTI over NNRTI resistant mutations and faster reversion to wild type HIV-1(614,615). Finally, no PI or INSTI resistance was observed in the naïve patients from 2007-2011, which reflects very limited use of these treatments in Uganda during this time period.

Following first-and second-line treatment failures, 97.8% and 81.9% of the HIV-1 sequences showed resistance to at least one ART drug, respectively (Fig. 19). Resistance and presence of drug resistant mutations was mainly observed in the NRTI and NNRTI drug classes following first line treatment failures due to exclusive use of these drug classes on cART initiation (Figure 19, Table 3). Due to complete absence of PI treatment in the first line treatment regimens, the PR coding region was not sequenced upon treatment failure.

Table 3. Frequency of mutations associated with reduced susceptibility to protease, reverse transcriptase, and integrase strand transfer inhibitors.

	PI [a] mutation (n)	NRTI [b] mutation (n)	NNRTI [c] mutation (n)	INSTI [d] mutation (n)
ARV-naive	none	E44D (4)	G190A (3)	M50I/L (29)
		T69D (5)	E138A (7)	L74I/M (5)
		K70R (7)	Y181C (6)	T97A/T (7)
		M184V (11)	K101E (3)	Others (1)
		M41L (3)	K238T (2)	
		L210W (2)	K103N (4)	
		D67N/G (6)	A98G (5)	
		K219Q/E (5)	V108I (2)	
		T215Y/I/F (7)	Others (7)	
		Others (5)		
First-line Failures	n.d.	E44D/E (16)	Y181V (5)	M50I/L (46)
		T69D/N (10)	E138A (3)	L74I/M (6)
		K70R (32)	K238T (6)	T97A/T (8)
		M41L (47)	H221Y (18)	E157Q (2)
		L210W (31)	V108I/V (40)	Others (1)
		K70KR (4)	H221HY (11)	
		V75V/I/M (45)	V179D/T (6)	
		A62AV (6)	P225H/P (8)	
		F77FL (2)	A98G/A (26)	

		K70KE (3)	M230L (6)	
		L210LW (4)	F227L (4)	
		M184V/M/I (131)	L100I (7)	
		D67/N/G (46)	Y188L (6)	
		L74V/L/I (18)	Y181C/V (49)	
		Y115F/Y (15)	E138E/A/G (5)	
		K65R/K (27)	K101P/H/E (37)	
		T215I/F/Y/T (71)	G190A/G/Q/S (52)	
		K219N/K/E/Q (42)	K103N/K/S (58)	
		Others (19)	Others (10)	
Second-line Failures	M46M/K/L (4)	K65R (5)	E138A (7)	M50I/L (34)
	I47A (5)	Y115F/Y (12)	Y181C (17)	L74I/M (9)
	V82A/F (19)	E44D (12)	K103N/K (28)	T97A/T (9)
	I84V (6)	T69D (6)	K101E (9)	
	L76V (5)	K70R (12)	K238T (4)	
	I54V (12)	M184V/M/I (57)	P225H (6)	
	L90M (2)	M41L/M (20)	A98G/A (14)	
	N88S 92)	L210W (10)	V108I/V (23)	
	V82S/C (2)	T215F/Y/I/T/S(62)	H221H/Y (7)	
	Others (1)	D67N/D (18)	M230L (3)	

		K219Q (9)	G190S/A/G (23)	
		T215I (3)	Y188L (5)	
		K219E (9)	H221HY (3)	
		Y115Y/F (11)	V179I/T/A (5)	
		F116Y (3)	K101E/H (16)	
		Q151M (4)	Others (9)	
		V75M/I (11)		
		K70K/R/E (8)		
		F77L (3)		
		L210LW (3)		
		L74I (7)		
		Others (11)		
RAL Failures	I54V/L (5)	M184V/M (8)	Y181C (5)	M50I/L (7)
	M46I/L (4)	K219E (2)	G190A (2)	L74I/M (7)
	V82F (2)	M41L (2)	K103N (4)	T97A/T (11)
	L76V (2)	E44D (2)	E138Q (3)	E138K/A (2)
	I84V (2)	Others (15)	Y188L (4)	E157Q (2)
	Others (5)		Others (11)	G163R (6)
				Y143R (3)
				N155H (6)
				Others (3)

[a]The number of primary resistance mutations in protease region of HIV-1 found in the study patients. [b]Primary resistant mutations which confer resistance to NRTIs. [c]Primary resistant mutations which result

in resistance to NNRTIs. [d]Primary resistant mutations which confer resistance to INSTIs. n.d, drug resistant mutations not determined. Others, drug resistant mutations with one frequency in each patient group.

However, previous studies in Uganda have confirmed the near absence of PR resistant mutations in first line treatment regimens involving two NRTIs and an NNRTI (578). PIs typically lopinavir/ritonavir (LPVr) or atazanavir/ritonavir (ATVr) were prescribed in nearly all second line treatments, hence the appearance of PI resistance upon failure. Despite the absence of NNRTIs in second- or third-line treatment with RAL, NNRTI resistance remained and was still the most common in these patients found in 75.5% and 49.0% patients respectively. Once PIs are administered, PIs are typically retained in the regimen even upon treatment failure and emergence of PI resistant mutations. As a consequence, we have little to no data on the potential loss or reversion of PI resistant mutations, except that PI resistance is still less frequent than NNRTI or NRTI resistance in newly infected or treatment naïve patients.

Unlike NNRTI, NRTI, and PI resistance, major drug resistance mutations (DRMs) to INSTIs were noticeably absent in N, FF, and SF, i.e., Y143R/C/H, Q148R/K/H/N, N155H, E92Q, E138A/K/T, G140S/A/C, S147G, T66A/I/K and R263K conferring resistance to RAL, EVG, and/or DTG. Nevertheless, we detected some minor IN mutations described as secondary or compensatory mutations: T97A/T (9.6%), M50I (6.7%), L74M/I (3.1%), E157Q (1.43%), V151I/A (2.0%) and G163R (2.0%) (Fig. 19, Table 4). L74M was observed in 0.8% of INSTIs naïve patients (N, FF, SF) and 9.8% of RF.

Table 4. HIV-1 infected patients failing on RAL-based regimen with primary and/or secondary (compensatory) INSTI mutations.

Primary/secondary mutations	n (%)	INSTI susceptibility		
		DTG	RAL	EVG
M50I	1(3.0)	S	S	S
M50I,L74I	1(3.0)	S	S	S
T97A	1(3.0)	S	P	P
T97A,G163R,L74M	3(8.8)	S	L	L
N155H	2(6.0)	P	H	H
N155H,T97A	1(3.0)	P	H	H
N155H,T97AT	1(3.0)	P	H	H
N155H,T97A,E157Q,L74I	1(3.0)	P	H	H
N155H,E157Q,G163R,M50L, L74I	1(3.0)	P	H	H
Y143R,T97A	2(6.0)	S	H	L
Y143R,T97AT,G163R	1(3.0)	P	H	I
Y143R,T97A,M50I,L74LM	1(3.0)	P	H	I
E138A,T97A,V151A	1(3.0)	P	I	I
E138K,G140A,S147G,Q148K	1(3.0)	H	H	H
T66A,T97A,G163R,L74M	1(3.0)	S	I	H

Secondary mutations (not included in the table) found in N, FF, and SF, were classified as follows; M50I was found in 12 (7.5%) of FF, 4 (3.3%) of SF, and 7 (8.0%) of N. L74M found in 1(0.8%) of SF, and 1 (1.1%) of N. T97A was found in 8 (5.0%) of FF, 9 (7.4%) of SF and 7 (8.0%) of N. E157Q was found in 2(1.2%) of FF, and 1 (1.1%) of N. In bold, the major INSTIs primary resistance mutations which confer resistance to INSTIs. Abbreviations: H, high level resistance, I, intermediate level resistance, L, low level resistance, S, susceptible genotype. Table 3. Frequency of mutations associated with reduced susceptibility to protease, reverse transcriptase, and integrase strand transfer inhibitors.

It is a polymorphic accessory mutation selected by RAL and EVG (616) and selected by DTG in previously RAL treated patients with DTG primary mutations. The M50I polymorphism was

observed in 6.2% of INSTIs naïve patients and 9.8% of RF, and increases resistance to DTG in combination with R263K to 5.6-fold in cell culture, though it does not increase the replication capacity of the virus. E157Q was found in 0.8% of INSTIs naïve and 5.8% of RF. It has been identified as a compensatory mutation for the N115H mutation, but also tends to increase susceptibility to DTG. T97A was found in 6.5% of INSTIs naïve and 29.4% of RF. T97A has been shown to pre-exist in 5%-10% of INSTIs naïve patients infected with subtype A virus. Additionally, T97A was previously co-selected in the presence of primary INSTIs DRMs by RAL in many clinical studies (423,617), and by DTG in treatment experienced patients with preexisting RAL associated resistance mutations (618). As expected, lack of INSTI resistance is attributable to absence of INSTI treatment in Uganda and most of sub-Saharan Africa. However, previous reports and data presented herein also indicate that natural polymorphisms conferring resistance to INSTIs are extremely rare in these subtype A and D isolates, also reported as rare in subtype B (619). Predicted DTG resistance was also absent in HIV-1 variants from 366 treatment naïve- or experienced patients (FL and SL treatment failures) (Fig. 19, Fig. 20).

INSTI resistance major mutations Y143R/S (0.9%), Q148K/R (0.47%), N155H (2.1%), E138A/K (0.7%), G140A (0.47%), T66A/TAIV (0.47%), and S147G (0.25%) (Fig. 19, Table 4) were only observed in RF.

However, only two patients (DR-206-12, DR-1059-17) in RF had genotypes with potential resistance to DTG (i.e., G140A, S147G, Q148K, E138K, and G140A, Q148R, E138A, and G163R respectively). In addition, R263K, the mutation most commonly associated with DTG resistance, was not observed. Over 47% RF had RAL and EVG resistance but only 23.5% were predicted to have weak and moderate resistance to DTG

3.6 Discussion

The Ministry of Health in Uganda has implemented the WHO "Treat All" recommendation which states that every person tested HIV-1 positive be started on treatment irrespective of his/her virological and immunological status. With increasing emergence of drug resistance in treatment naïve population in LICs (524,615,620,621), and the number of patients on cART increasing, HIV-1 drug resistance prevalence will inevitably also rise. More potent antiretroviral drugs, such as DTG, have shown to be active in treatment-experienced patients. More importantly, resistance to DTG seems to be infrequent in cART-naïve individuals treated with this integrase inhibitor (622). The purpose of the study was to screen for drug resistance and possible treatment with DTG in treatment naïve patients and those experiencing virologic failure during first, second-, and third-line cART in Uganda. To our knowledge, this is the first study to look at INSTIs associated drug resistance in both cART naïve and experienced patients in Uganda and most other countries in sub-Saharan Africa.

Among minor INSTI resistance mutations, T97A mutation was observed in both INSTIs naïve and RF and has been shown to reduce sensitivity of virus to INSTIs and/or rescue viral fitness in combination with Y143C/R, Q148+G140S, or N155H. However, a previous study shows T97A doesn't significantly reduce susceptibility to INSTI with up to 94% and 97% viral suppression achievable in HIV-1 patients with preexisting and emerging T97A respectively (623). The prevalence of the M50I polymorphism observed in INSTI-naïve patients is lower compared to the 10% found in patients infected with HIV-1 subtype B virus, which shows variation in the evolution of INSTI-associated mutations in different viral subtype populations. A relatively small number of patients were infected with viruses carrying the E157Q mutation, which has shown to increase susceptibility to DTG (624). However, the combination of E157Q and R263K increases DTG resistance by 10-fold (624).

We found neither of the two rare mutations associated with DTG resistance, R263K and G118R. R263K has been identified in both clinical samples and cell culture assays (429,435) and G118R in cell culture tests. The major mutation pathways for RAL, Y143R/C, Q148R/K/H and N155H(419) were not found in INSTIs naïve patients in agreement with previous study done in treatment naïve patients in South Africa (625). In RF, 18 (35%) of patients had Y143R/S,

Q148R/K, G140A, E138A, S147G, T66A/TAIV, and N155H INSTI major mutations which could explain the virological failure observed in these patients.

This study shows that accumulation of INSTIs DRMs at positions, G140, S147, Q148, and E138 after RAL failure can potentially predict the potential loss of susceptibility of the virus to DTG as seen previously (626). For example, we found two individuals infected with a virus carrying the Q148K/R resistant mutation, which when present alone moderately reduces RAL and EVG susceptibility while having a minimal effect on DTG susceptibility; however, in combination with G140S/A/C and/or E138K/A, it may reduce DTG susceptibility up to 10-fold (607,627). In RF, N155H had highest frequency 9 (17.6%) compared to other major INSTIs DRMs which may be due to early selection of this resistant mutation under RAL pressure as observed previously in a phase II study looking at long term efficacy and safety of RAL in patients with limited treatment options(628). Among the six major mutations which reduce susceptibility to EVG, T66I, E92Q, T97A, S147G, Q148K and N155H (424), only substitution E92Q was not selected by the virus in the study patients which could be due to cross resistance as EVG is currently unavailable in the country.

Based on our analyses of 417 Ugandan HIV-1 pol sequences, DTG would possibly be effective at any stage of cART treatment in sub-Saharan Africa. DTG has become the preferred drug for the majority of new FL or salvage treatments in HICs. In our Ugandan cohort, we did not observe a higher frequency of mutations conferring DTG or any other primary INSTI resistance mutations. We observed a high frequency of NNRTI resistance in FF (96.4%), and in patients who remain on treatment but who have not received NNRTI for years, SF (75.5%) and RF (49.0%). This observation complements the fact that over 50% of treatment failures in Uganda retain NNRTI resistant virus and often for years following the last dose of nevirapine (NVP) or efavirenz (EFV). In contrast, the frequency of NRTI and PI resistance is lower in all of these HIV-1 infected groups in Uganda. With the UNAIDS/WHO 90:90:90 goals, continued use of NNRTIs (EFV or NVP) may help increase access to treatment since these drugs are readily available in this setting but may not impact treatment outcomes due to associated high drug resistance, high pill burden, and poor tolerability profiles. However, to achieve continued viral suppression in 90% of treated individuals, we should abandon the continued use of NNRTIs (NVP or EFV) in first line treatment and strongly advocate for the use of second generations INSTIs, such as DTG or even Bictegravir,

in first line treatment regimens in lower income countries (LICs), such as Uganda and other East African countries.

Chapter 4

4 Accumulation of integrase strand transfer inhibitor resistance mutations confers high-level resistance to Dolutegravir in non-b subtype HIV-1 strains from patients failing Raltegravir in Uganda

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All of the experimentation on this study was performed and/or supervised by Emmanuel Ndashimye

4.1 Preface

Raltegravir is commonly used as a salvage therapy in most LMICs in combination with darunavir and NRTIs. Though RAL is sparingly used in Uganda, over 50% of patients failing third line therapy harbor RAL/EVG associated DRMs with 4% predicted to harbor DTG resistance. DTG has a higher barrier to resistance profile and for that treatment failure in DTG naïve or experienced patients is rare. Indeed, from our previous study, DTG resistance was only predicted in patients with multiple INSTIs associated DRMS failing RAL-based regimen. The prediction was based on Stanford HIV-1 drug resistance database interpretation and therefore it was necessary to confirm these results by carrying out phenotypic assays on recombinant viruses from patient-derived HIV-1 integrases.

E.N, Y.L, A.M, C.T, P.S performed drug resistance assays and experimentation in this article and E.N, M.A, A.P, A.S.O, R.M.G, M.E.Q-M performed data analyses. I.N, F.K, C.K recruited all the patients for this study. E.J.A and M.E.Q-M procured the funding, and E.J.A designed, supervised, and guided the overall direction of the study as the principal investigator.

4.2 Abstract

Objectives

Increasing first-line treatment failures in low-and middle-income-countries (LMICs) has led to increased use of integrase strand-transfer inhibitors (INSTIs) such as dolutegravir (DTG). However, HIV-1 susceptibility to INSTIs in LMICs especially with previous raltegravir (RAL) exposure is poorly understood due to infrequent reporting of INSTI failures and testing for INSTI drug resistant mutations (DRMs).

Methods

A total of 51 non-subtype B HIV-1 infected patients failing third-line treatment regimen (RAL-based) in Uganda were initially selected for the study. DRMs were detected using Sanger and deep sequencing. HIV-1 integrase genes of 13 patients were cloned and replication capacities (RCs) and phenotypic susceptibilities to DTG, RAL, and elvitegravir (EVG) were determined with TZM-bl cells. Spearman's correlation coefficient was used to determine cross resistance between INSTIs.

Results

INSTIs DRMs were detected in 47% of patients. HIV-1 integrase-recombinant virus carrying one primary INSTI DRM (N155H or Y143R/S) was susceptible to DTG but highly resistant to RAL and EVG (>50-fold). Two patients with E138A/G140A/Q148R/G163R or E138K/G140A/S147G/Q148K displayed highest reported resistance to RAL, EVG, and even DTG. The former multi DRM virus had wild type RC whereas the latter had lower RCs than wild type.

Conclusions

In HIV-1 subtype A and D infected patients failing RAL and harboring INSTIs mutation DRMs, there is high level resistance to EVG and RAL. More routine monitoring of INSTI treatment may be advised in LMICs considering multiple INSTI DRMs may have accumulated during prolonged exposure to RAL during virologic failure, leading to high level INSTI resistance, including DTG resistance.

4.3 Background

HIV-1 integrase strand transfer inhibitors (INSTIs), the latest class of ART, have changed the HIV-1 treatment landscape around the world. The second generation INSTI dolutegravir (DTG) has a higher genetic barrier to resistance and has proved to be an effective INSTI when used in combination therapy for treatment-naïve as well as individuals harboring viruses resistant to raltegravir or elvitegravir (318,444). Under 2018 WHO guidelines, a DTG-based regimen is preferred for first-line ART for all adults and adolescents except for those women of childbearing age who wish to become pregnant. DTG is also the preferred second-line drug of choice for those failing NNRTI-containing treatment regimens (629). For children >4 weeks of age, DTG is favored for second-line treatments following failure to NNRTIs or PI containing ART (629). Following FDA approval in 2013, and prior to 2014, DTG was rarely administered in low-income and middle-income countries (LIMCs) but with the rollout of TDF+3TC+DTG at a cost of US $75 annually per patient-year (630), over 4 million patients are now receiving a DTG- containing regimen (631), eclipsing the number of patients receiving DTG in the high income countries (HICs). In Uganda, the focus site of this study, generic DTG is now part of the preferred first-line regimen (TDF+3TC+DTG) in all adults (525).

RAL performs pivotal role as salvage and pediatric treatment and EVG can be used in ART naïve patient in Uganda and other LMICs. However, RAL and EVG resistance has been reported but mostly in HIV-1 subtype B due to early use of INSTIs (632). In addition, there is infrequent reporting of INSTI failures and testing for INSTI drug resistant mutations in non-B subtypes especially subtype A and D. Switching patients to salvage treatment in Uganda is done under discretion of a committee by the ministry of health which rely on resistance predictions from HIV-1 drug resistance databases (525). However, there appears to be subtype specificity associated with resistance to INSTIs with some mutations. For example, substitutions at Q148 of HIV-1 integrase which confers resistance to RAL, EVG, and cross resistance to DTG appears more in subtype B than non-B subtypes (633). Thus, it becomes imperative to assess the impact of RAL and EVG associated mutations in-vitro to further elucidate on impact of these mutations in subtype A and D integrases.

Despite the rapid and large scale roll out of DTG in LMICs, there is very limited data on the susceptibility of non-subtype B HIV-1 to INSTIs (592,633), including cross-resistance to DTG conferred by raltegravir-resistant variants HIV-1 emerging in non-B HIV-1 subtype infected patients failing raltegravir-based regimens (633). In addition, increasing spread of HIV-1 non-B subtypes in regions where subtype B previously predominated such as Europe and America (634–636), calls for the urgent need for more genotypic drug susceptibility testing for INSTI resistance.

Even with the use of generic DTG for first-line ART in LIMCs, treatment failures to the NNRTI-based and subsequent boosted PI-based regimens are still followed by raltegravir-based ART. Failure to this third-line treatment in LMICs leaves patients with few options since entry inhibitors, i.e., enfuvirtide- and maraviroc, are not readily available. In our previous study, we found over 50% of patients failing on raltegravir-based therapy in Uganda harbored raltegravir-associated DRMs (540).

In this study, non-B HIV-1 integrase (IN) chimeric viruses derived from 11 Ugandan patients failing a raltegravir-based third-line regimen showed significant resistance to raltegravir and elvitegravir but remained susceptible to DTG when the “common” raltegravir resistance mutations were present: N155H, Y143R/S. However, viruses harboring three, or four resistance mutations (i.e., E138K/A, G140A, S147G, Q148K/R, and G163R) showed high-level resistance to all INSTIs, and only one had significantly impaired replicative rates.

4.4 Materials and methods

Samples for the study

Samples were collected from the WHO, College of American pathologist (CAP) and National Institutes of Health-virology quality assurance (NIH-VQA)-accredited center for AIDS Research (CFAR) laboratory of the Joint Clinical Research Center (JCRC) in Kampala, Uganda. The patient database in the CFAR laboratory was used to retrieve the patient demographic, medical, and treatment histories. A total of 60 plasma samples were collected from patients failing raltegravir-based third-line regimens. Virologic failure was defined by a viral load above 1,000 copies/ml and/or $CD4^+$ T cell counts below 250 cells/mm^3 defined immunological failure. Written consent was obtained from all patients prior to sample storage. Ethical clearance was obtained from the IRBs at the JCRC and University hospitals Cleveland medical center/Case Western reserve university (EM-10-07 and 10-05-35).

RNA extraction and PCR amplification

HIV-1 viral RNA was extracted from plasma using a QIAamp viral RNA Mini Kit (Qiagen) and HIV-1 IN-coding region amplified using a Superscript III single RT-PCR system (Thermo Fisher Scientific) as previously described, (540) (Table 5).

Sanger sequencing and sequence analysis

The HIV-1 IN coding regions were amplified and analyzed using Sanger sequencing as previously described (540). Briefly, a quantified and purified PCR product was sequenced to cover the full length of the HIV-1 IN (1–288 amino acids) (Table 5). Sequences were exported and analyzed in RECall (beta v3.02) program as recommended by the WHO (606).

Table 5. List of primers used in the study

Name	5'-3' sequence	Function
RTA9F	TATGGGGAAAGACTCCTAAATTTA	RT/PCR (F)
3Vif	AGCTAGTGTCCATTCATTG	RT/PCR (R)
Vif 3 reverse1	GTCCTGCTTGATATTCACACC	Nested PCR, Sanger (R)
INTFEXT1	AGAAGTAAACATAGTAACAGACTCACA	Nested PCR, Sanger (F)
INTREXT	AATCCTCATCCTGTCTAC	Sanger (R)
INTF1B	AGGTCTATCTGGCATGGGTACC	Illumina library (F)
INTR1B	GATTGTAGGGAATTCCAAATTCCTGCT	Illumina library (R)
INTF2B2	CAGGAATTTGGAATTCCCTACAATCCCC	Illumina library (F)
INFR2B4	TGTCTATAAAACCATCCCCTAGCTTTCCC	Illumina library (R)
Forward overhang: 5'	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	Illumina library (F)
Reverse overhang: 5'	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	Illumina library (R)
INTF-VECT2	AGTGCTGGAATCAGGAAAGTACTATTTTTAGATGGAATAGATAA	pREC_NFL_IN/URA3 overhang (F)
INTR-VECT2	TTTTACTAATCTTTTCCATGTGTTAATCCTCATCCTGTCTACT	pREC_NFL_IN/URA3 overhang (R)

RT, reverse transcription; F, forward; R, reverse

The Stanford HIV-1db Sierra web service algorithm v8.3 was used to predict resistance phenotype. (637)

Library preparation and deep sequencing

An amplicon-based deep sequencing method was used to detect variants and confirm presence of DRMs originally identified by Sanger sequencing (Table 5). Briefly, two overlapping PCR products spanning full-length of HIV-1 IN were purified using (Agencourt AMPure XP; Beckman Coulter) and quantified using (Quant-iT Picogreen dsDNA assay kit; Thermo scientific).

Table 6. HIV-1 drug resistance interpretation and subtypes of all study patients

Sample ID	IN Major mutation	IN Accessory mutation	Subtype	DTG Score	DTG[a]	EVG Score	EVG[b]	RAL Score	RAL[c]
UG11	N155H	None	D	10	P	60	H	60	H
UG23	E138A	T97A,V151A	A	10	P	55	I	40	I
UG35	Y143R	T97A,M50I,L74IM	A	5	S	25	L	70	H
UG39	T66A	T97A,G163R	A	0	S	85	H	40	I
UG42	N155H	E157Q,G163R,M50L,L74I,V151I	D	10	P	85	H	85	H
UG45	N155H	T97A,E157Q	A	10	P	80	H	80	H
UG103	None	None	A	0	S	0	S	0	S
UG110	None	None	A	0	S	0	S	0	S
UG111	Y143R	T97A	A	10	P	30	I	70	H
UG115	None	None	A	0	S	0	S	0	S
UG119	None	None	A	0	S	0	S	0	S
UG138	N155H	T97A,L74I	A	10	P	70	H	70	H
UG160	None	T97A	A	0	S	10	P	10	P
UG171	None	None	D	0	S	0	S	0	S

UG201	None	None	A	0	S	0	S	0	S
UG202	None	None	D	0	S	0	S	0	S
UG206	E138K,G140A,S147G,Q148K	None	D	80	H	180	H	120	H
UG252	None	None	D	0	S	0	S	0	S
UG282	None	None	A	0	S	0	S	0	S
UG294	None	None	A	0	S	0	S	0	S
UG296	None	None	D	0	S	0	S	0	S
UG342	None	None	A	0	S	0	S	0	S
UG370	None	T97A,G163R	A	0	S	25	L	25	L
UG372	None	None	A	0	S	0	S	0	S
UG419	None	None	D	0	S	0	S	0	S
UG479	None	None	A	0	S	0	S	0	S
UG481	Y143R	TA97TA,G163R	A	10	P	45	I	85	H
UG485	None	T97A,G163R	A	0	S	25	L	25	L
UG487	N155H	T97TA	A	10	P	70	H	70	H
UG533	None	None	D	0	S	0	S	0	S

UG537	N155H	None	A,D	10	P	70	H	70	H
UG70	Y143R	T97A	A	5	S	25	L	70	H
UG91	None	T97A,G163R	A	0	S	25	L	25	L
UG93	None	None	A	0	S	0	S	0	S
UG1044	Y143S	T97A	A	5	S	35	I	80	H
UG1059	E138A,G140A,Q148R	G163R	A	80	H	135	1I	135	1I
UG1060	None	None	A	0	S	0	S	0	S
UG1062	None	None	A	0	S	0	S	0	S
UG1179	N155H	None	A	10	P	60	H	60	H
UG1182	None	T97A,D232N	A	0	S	20	L	20	L
UG172	T66TAIV	T97A	A	5	S	70	H	25	L
UG200	None	None	A	0	S	0	S	0	S
UG239	None	None	A	0	S	0	S	0	S
UG333	None	None	D	0	S	0	S	0	S
UG345	None	None	A	0	S	0	S	0	S
UG432	N155H	T97A,E157O	A	10	P	80	H	80	H
UG563	None	None	C	0	S	0	S	0	S
UG56	None	None	A	0	S	0	S	0	S
UG63	N155NH	T97TA,P142PT	D	10	P	70	H	70	H
UG97	None	None	A	0	S	0	S	0	S
UG119	None	None	A	0	S	0	S	0	S

[a,b,c] HIV-1 susceptibility to DTG, EVG, RAL respectively. Sanger and Illumina sequences were predicted for resistance using Stanford HIV-1 database v8.3 (https://HIV-1db.stanford.edu/HIV-1db/by-sequences/). DTG, dolutegravir; EVG, elvitegravir; RAL, raltegravir; H, high level resistance; I, intermediate resistance; L, low level resistance; P, potential resistance; S, susceptible; IN, HIV-1 integrase.

The barcodes were added using Nextera XT index Kit v2 (Illumina) (Table 4) and paired-end sequencing done on a MiSeq instrument (Illumina). Analysis was performed with MiSeq Reporter analysis software version 2.6 (Illumina) and drug resistance interpretation was done using Stanford HIV-1db Sierra web service algorithm v8.3 (637). Samples confirmed by deep sequencing to harbor INSTIs associated DRMs (n=11) or having no known INSTIs associated DRMs (n=2) were selected for phenotypic testing on DTG, raltegravir and elvitegravir (Table 6, Table 7).

Table 7. Virological and clinical characteristics of patients with INSTIs associated mutations in the study

Sample ID[a]	*cART*[b]	*Viral load c/ml*[c]	*Subtypes*[d]	*Resistance mutations*[e]
UG1179	LPVr/RAL	ND	A	N155H
UG11	ATVr/RAL	ND	D	N155H
UG537	LPVr/RAL	255,641	A/D	N155H
UG138	DRVr/RAL	275,059	A	N155H, T97A, L74I
UG42	LPVr/RAL	155,982	D	N155H, E157Q, G163R, M50L, L74I, V151I
UG35	DRVr/RAL	2,293,840	A	Y143R, T97A, M50I, L74IM
UG1044	TDF/3TC/DRVr/RAL	29,200	A	Y143S, T97A
UG481	TDF/FTC/DRVr/RAL	3,914	A	Y143R, TA97AT, G163R
UG23	LPVr/RAL	850	A	E138A, T97A, V151A
UG1059	TDF/3TC/LPVr/RAL	14,200	A	E138A, G140A, Q148R, G163R
UG206	LPVr/RAL	1,515,000	D	E138K, G140A, Q148K, S147G
UG14	ART naive	3008	A	None
UG98	ART naive	ND	D	None

The HIV-1 subtype was predicted using SCUEAL subtype classification algorithm. Viral loads were assayed using Abbott m2000sp/rt or Roche COBAS Amplicor Monitor ultrasensitive tests, v1.5. In bold, major INSTIs resistance mutations [a] The patients accession numbers tested for INSTI resistance. [b]Description of the ART combinations in the study [c]The patient viral loads (copies/ml) dThe predicted subtype of study patients [e]the resistance mutations detected in study patients cART, combined antiretroviral therapy; ND, not determined; INSTI, integrase strand transfer inhibitor; ATVr, atazanavir/r; DRVr, darunavir/r; LPVr, lopinavir/r; RAL, raltergravir; 3TC, lamivudine; TDF, tenofovir; FTC, emitricabine.

HIV-1 subtype classification and detection of recombination forms was done using SCUEAL program (608).

Cells and antiviral compounds

TZM-bl, U87.CD4.CXR4, and HEK293T cell lines were obtained through the AIDS Research and Reference Reagent program, division of AIDS, NIAID, NIH. TZM-bl cells, and HEK293T were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin-streptomycin. U87.CD4.CXR4 cells were maintained in DMEM supplemented with 10% FBS, 100 µg/ml penicillin-streptomycin, 300 µg/ml G418, and 1 µg/ml puromycin (Invitrogen, Carlsbad, CA). All cell lines were subcultured every 3-4 days at 37°C under 5% CO_2. The TZM-bl cells had reporter luciferase and β-galactocidase reporter genes which can be activated by

expression of HIV-1 tat. Elvitegravir was obtained from (Gilead Sciences, Inc., Foster City, CA), raltegravir and DTG (NIH).

Construction of HIV-1 IN chimeric viruses

HIV-1 full length IN PCR products were recombined into near full-length HIV-1 (pREC_NFL_IN/URA3) vector using transfected *Saccharomyces cerevisiae* MYA-906 cells (ATCC) based on yeast homologous recombination-gap repair system (638). Plasmids were extracted from the yeast cells using phenol/chloroform (Thermo Fisher scientific) and transformed into electrocompetent *Escherichia coli* Stbl4 cells (Thermo Fisher scientific). Plasmids were extracted from the bacteria using Qiagen miniprep kits (Hilden, Germany). The presence of mutation (s) in generated plasmid was confirmed by Sanger sequencing. pREC_NFL_INT plasmids were cotransfected into HEK 293T cells (3×10^4 cells/well) along with the complementing plasmid pCMV_cplt using Fugene 6 reagent (Promega, Madison, WI) (638). Produced heterodiploid virus particles containing one copy of the pREC_NFL_INT and cplt HIV-1 RNAs were further propagated on U87.CD4.CXCR4 cells to produce a complete HIV-1 genome. Following virus propagation, viral RNA was re-sequenced to confirm the presence of the various DRMs.

INSTIs resistance assays in TZM-bl cells

The susceptibility of patient-derived viruses to DTG, raltegravir and elvitegravir was determined using short-term resistance assays with TZM-bl cells. Briefly, 20,000 cells were seeded to each well of 96-well plate and infected with controls, NFL4-3, UG14, UG98, and mutant viruses in presence of 10-fold dilutions of DTG, raltegravir, or elvitegravir (100 µM to 10^{-8} µM) and DEAE-dextran (1mg/ml). The amount of virus added to each well was normalised to 0.01 MOI based on infectious titre. After 48 hr incubation at 37°C and 5% CO_2, the infectivity of viruses was quantified using X-gal as previously described (639). The stained colonies were counted using Cytation 5 plate reader (BioTek USA) and confirmed by manual counting using a fluorescent microscope. Drug sensitivity curves were generated using nonlinear regression curve fitting features of GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA). Drug resistance was expressed as fold change (FC) in effective concentration 50 (EC50) between

controls and mutant viruses basing on at least two sets of experiments each performed in quadruplicates.

X-gal staining assay

The X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (40 mg/ml) substrate was used to detect β-galactocidase enzyme expression from infected cells (639). The TZM-bl cells infected for 48 hrs were fixed with glutaraldehyde (0.2%) and formaldehyde (0.8%). The cells were sequentially stained with potassium ferrocyanide (0.2M), potassium ferricyanide (0.2M), magnesium chloride (2M), and X-gal substrate, and then incubated for 2 hours at 37°C.

Infectivity assay in TZM-bl cells

Infectivity of HIV-1 IN chimeric mutant viruses were determined using short-term infectivity assay in TZM-bl cells. Briefly, 20, 000 TZM-bl cells in presence of DEAE-dextran (1mg/ml) were infected with increasing concentration of either mutant or controls. Cells were fixed, stained, and β-galactosidase expression was measured using X-gal protocol as described above. The fold decreases in infectivity were expressed as percentage of relative decrease in area under the curve, amount of virus needed for TZM-bl cells to produce the maximal level of β- galactosidase in an infection.

Statistical analyses

Statistical analyses were performed using non-linear regression in GraphPad Prism 8.1.2 (GraphPad Software, La Jolla, CA). The level of cross resistance was analysed using Spearman's rank order test.

4.5 Results

Samples and patient demographics

Samples were obtained from 60 patients failing a third-line raltegravir-based in Uganda. Of them, 51 (85%) were successfully amplified and sequenced by Sanger and deep sequencing (540). INSTI-associated DRMs were detected in 24/51 (47%) patient samples. Eleven samples with INSTIs resistance mutations were subsequently selected for phenotypic assays. Of the 11 samples selected for phenotypic studies, 8 of 11 were obtained from individuals on dual therapy (protease inhibitor [PI] + raltegravir) while the other 3 were treated with triple therapy (i.e., NRTI + PI + raltegravir). The average viral load count was 454,669 copies/ml (range 850 -2,293,840) (Table 7). Seven of 11 (64%) viruses were classified as HIV-1 subtype A, 27% subtype D (3/10) and 1 sample had a subtype A/D recombinant (Table 6, Table 7). Two patient IN with no INSTI-associated DRMs, UG98 (subtype D), UG14 (subtype A), as well as NL4-3 HIV-1 (subtype B) where included in phenotypic assays as reference strains (Table 7).

As previously published, (540) nearly 5% (2/51) of the patients failing a raltegravir-containing therapy harbored virus with multi-DRMs to INSTIs, i.e the subtype A UG1059 with E138A/G140A/G163R/Q148R and subtype D UG206 with E138K/G140A/S147G/Q148K. These were selected for cloning and phenotypic assays along with other 9 with INSTIs DRMs. Whereas Q148H only emerges in context of G140S mutants in subtype B, the Q148R/K appears associated with G140A in non-B subtypes. Overall, N155H was the predominant INSTI DRM found in 9 of 51 patients. For this study, three IN regions with only N155H (UG1179, UG11, and UG537) and two patients' IN containing N155H with secondary mutations, L74I/T97A or M50L/L74I/V151I/E157Q/G163R (respectively) were cloned for phenotypic analyses (Table 6, Table 7). Along with these seven, other three had Y143R plus secondary mutations M50I/L74IM/T97A, (UG35), or T97AT/G163R, (UG481), and Y143S plus T97A (UG1044). One patient had E138A, T97A, V15IA mutations (Table 6, Table 7).

N155H and Y143R/S emergence during raltegravir treatment in subtype A and D confer high level resistance to raltegravir and elvitegravir but not to DTG

The N155H mutation in HIV-1 subtype B confers significant level resistance to both raltegravir and elvitegravir. In drug susceptibility assays, the three subtype D and circulating recombinant form A/D patient derived recombinant viruses (UG11, UG537 and UG1179) carrying only N155H had a 10- to 19-fold decrease in susceptibility to raltegravir, 63- to 78-fold to elvitegravir, but only 1.3-fold change to DTG susceptibility when compared to susceptibility of wild type NL4-3 (Fig. 21, Fig. 22, Fig. 23). Slightly higher levels of resistance were observed with these N155H viruses when compared to the wild type subtype D or A IN cloned into NL4-3.

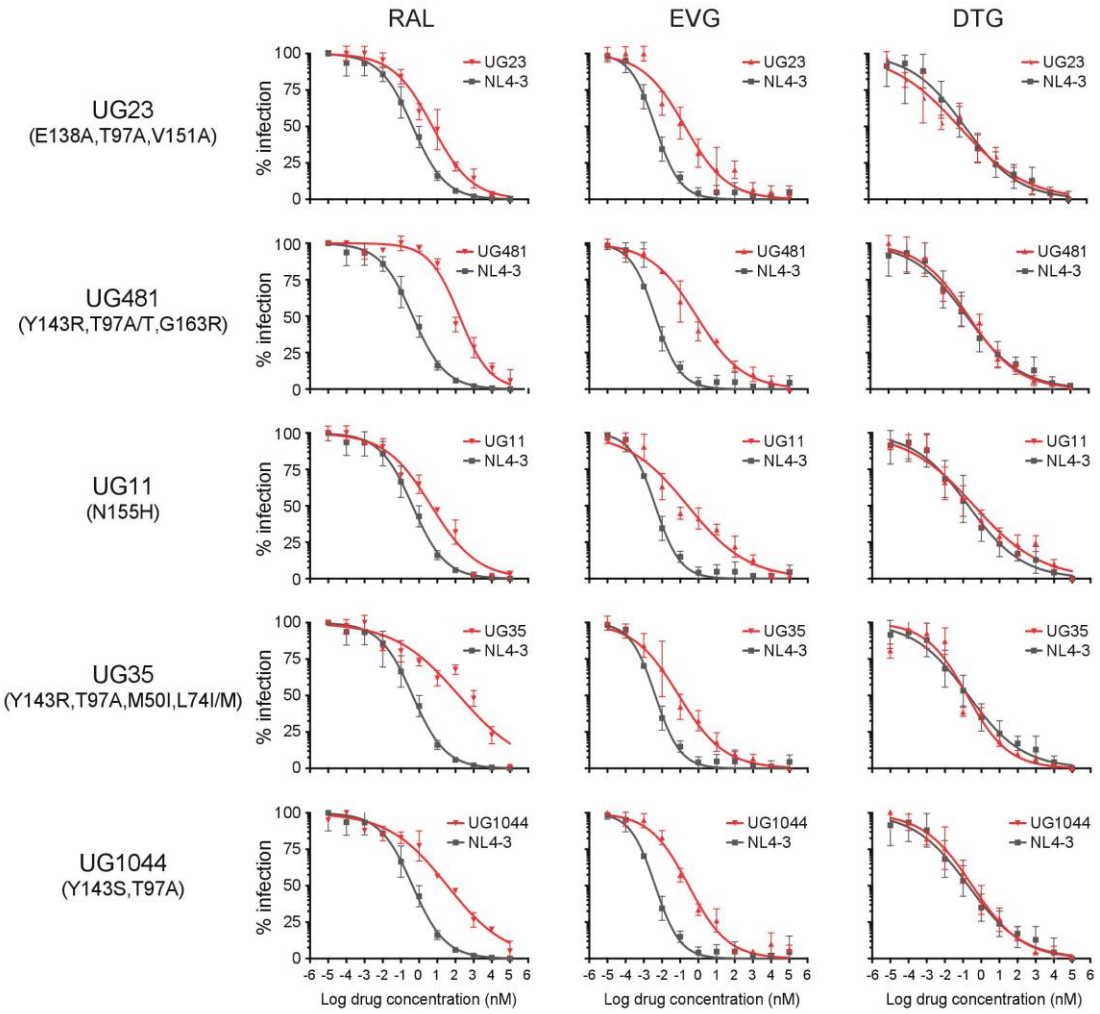

Figure 21.The susceptibility of viruses with drug resistant mutations to INSTIs. The log values of drug concentrations were plotted against percentage of infections in TZM-bl cells detected by x-gal assay. The colonies were counted using elispot and they are from 2-3 independent experiments each run in quadruplets. The change in EC_{50} relative to wild type (NL4-3) is shown. Ral: raltegravir, evg: elvitegravir, dtg: dolutegravir.

When L74I and T97A was found in addition to N155H in a subtype A IN, there was no effect on susceptibility to DTG (FC 1.57-fold change or FC) but increased resistance to raltegravir (FC 39) and elvitegravir (FC 48) was evident (when compared to the wild type NL4-3).

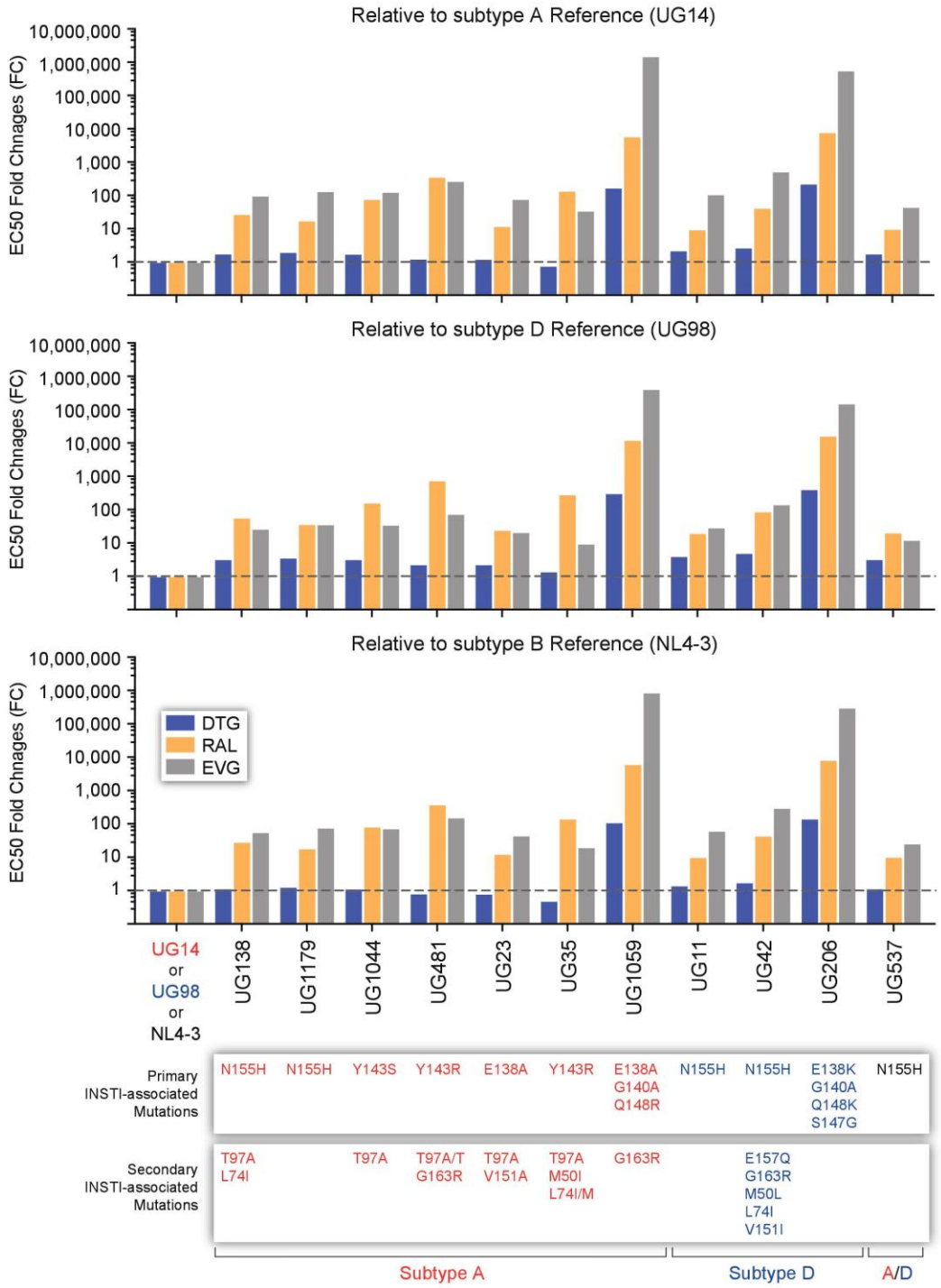

Figure 22.The susceptibility of mutants by subtype. The fold change values in EC_{50} of DTG, RAL and EVG relative to each subtype was determined in short term infection assay in TZM-bl cells. Each value represents the mean fold change of EC_{50} from 2-3 independent experiments each done in quadruplets.

Presence of secondary mutations, E157Q, G163R, M50L, L74I and V151I in addition to N155H in the subtype D integrase resulted in a low 2.8-fold resistance to DTG but high-level resistance to elvitegravir (FC 70) and raltegravir (FC 56).

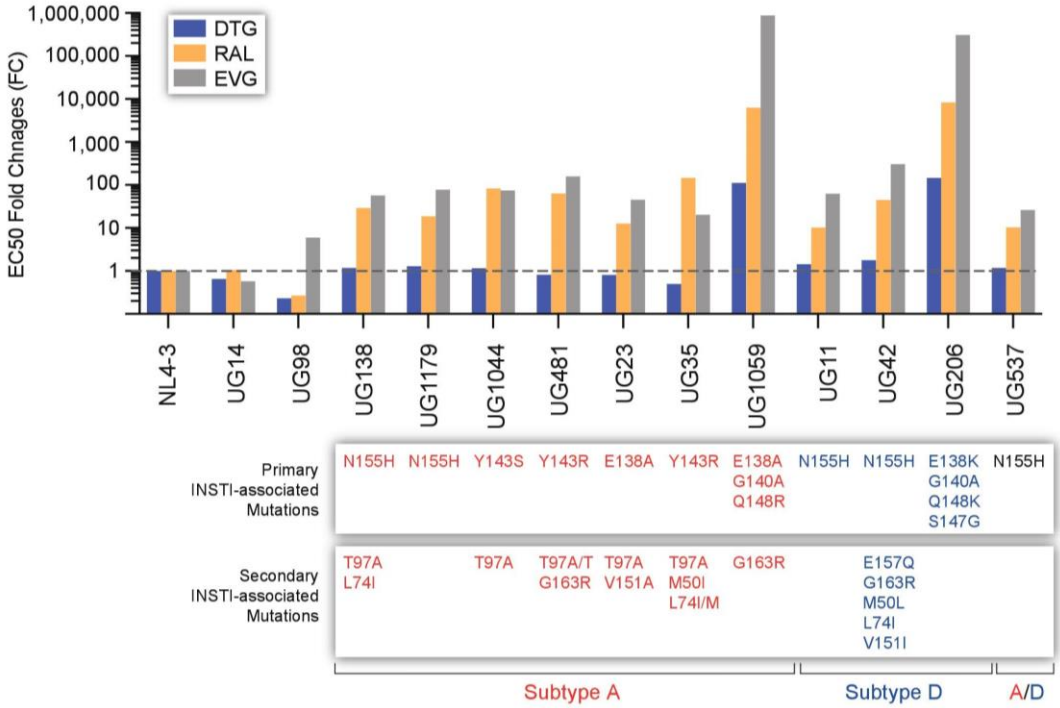

Figure 23.The susceptibility of HIV-1 mutants relative to NL4-3 strain. The fold change values in EC_{50} of DTG, RAL and EVG relative to NL4-3 was determined in short term infection assay in TZM-bl cells. EC_{50} fold changes of wild type A, D relative to NL4-3 are also shown. Each value represents the mean fold change of EC_{50} from 2-3 independent experiments each done in quadruplets.

The Y143R/S mutation only emerged in HIV-1 subtype A infected patients and conferred slightly higher levels of resistance to raltegravir than to elvitegravir in subtype A HIV-1. The T97A, M50I, and L74I/M secondary mutations had minimal or no impact on INSTI resistance. Susceptibility to DTG was not affected by Y143R/S with the other secondary mutations (Fig. 21, Fig. 22, Fig. 23).

Multiple INSTI DRMs with secondary mutations confers high level resistance to DTG, raltegravir and elvitegravir

Q148H/K/R alone typically has minimal effect on DTG susceptibility. In patients failing a raltegravir-based treatment in Uganda, the Q148R mutation in subtype A HIV-1 was found in combination with primary INSTI DRMs, E138A, G140A, and in subtype D, a Q148K with E138K, G140A, and S147G. With the IN from both patients, the chimeric viruses were highly resistant to elvitegravir, raltegravir (both >1000-fold), and DTG (>100-fold) (Fig. 22, Table 8, Fig. 23).

Table 8. The mean EC50 of different HIV-1 integrase strand transfer inhibitors against recombinant viruses in the study

	DTG		RAL		EVG	
Resistance mutations	EC50	95% CI (nM)	EC50	95% CI (nM)	EC50	95% CI (nM)
Wild type	0.34	0.1118 to 0.3429	0.48	0.3249 to 0.5906	0.00	0.003087 to 0.005806
N155H,T97A,L74I	0.40	0.1754 to 0.5453	13.86	6.424 to 43.5	0.27	0.00342 to 0.008193
N155H	0.44	0.1486 to 1.385	8.91	4.937 to 28.93	0.37	0.08511 to 0.9288
N155H	0.40	0.1649 to 0.5544	4.96	2.704 to 5.49	0.12	0.09238 to 0.2315
N155H	0.49	0.1883 to 0.8704	4.84	2.768 to 7.939	0.30	0.1233 to 0.5305
Y143S,T97A	0.39	0.1974 to 0.5412	39.66	17.72 to 71.01	0.35	0.1513 to 0.6432
Y143R,T97AT,G163R	0.28	0.1423 to 0.4362	183.75	99.82 to 262.4	0.75	0.4802 to 1.287
E138A,T97A,V151A	0.27	0.03887 to 0.2279	6.02	3.606 to 8.123	0.22	0.09659 to 0.3427
Y143R, T97A,M50I,L74IM	0.17	0.08362 to 0.3347	69.36	30.09 to 100.3	0.10	0.05267 to 0.1333
N155H,E157Q,G163R,M50L,L74I,V151I	0.60	0.1807 to 1.629	21.27	5.334 to 57.99	1.45	0.3769 to 2.698
E138A,G140A,Q148R,G163R	37.88	11.88 to 120.5	2996.03	2699 to 17863	4180.00	3085 to 5257
E138K,G140A,S147G,Q148K	49.67	27.61 to 87.55	4002.00	2624 to 6060	1550.90	1107 to 4491
None	0.22	0.1172 to 0.3252	0.50	0.3466 to 5.199	0.00	0.001148 to 0.00665
None	0.08	0.04878 to 0.1747	0.13	0.05141 to 0.2932	0.03	0.009819 to 0.03532

DTG, dolutegravir; RAL, raltegravir; EVG, elvitegravir; EC50, effective concentration 50; CI, confidence interval

Finally, chimeric virus from HIV-1 subtype A infected patient with secondary mutations T97A, V151A, and E138A had resistance to elvitegravir (45-fold) and low-level resistance to raltegravir (12-fold) but wild type sensitivity to DTG (Fig. 21, Fig. 22, Fig. 23).

For all the chimeric viruses studied for drug susceptibility, the IN genes of HIV-1 subtype A and D infected patients containing INSTI DRMs were cloned into a subtype B backbone. Despite the concerns with complementation compatibility, the same level of resistance with these IN chimeric viruses was observed when compared to wild type NL4-3 or the NL4-3 containing the wild type subtype A and D IN coding regions (Fig. 24).

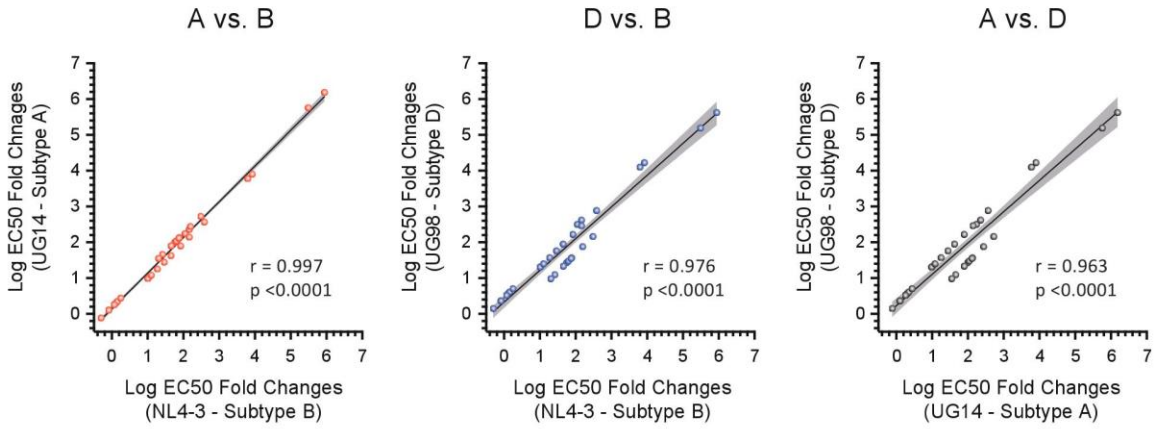

Figure 24. Effects of IN complementation in an NL4-3 chimeric virus. The correlation of fold change values relative to subtype A, D, and nfl4-3. Each value represents the fold change of means from each subtype. Correlation was determined using spearman's rank order with p=0.001 significant.

INSTI DRMs can impair replicative capacity regardless of subtype

Using TZM-bl cells replication capacity (RC) of these IN chimeric HIV-1 (all with NL4-3 backbone) were compared to wildtype NL4-3, UG14, and UG98 references. All the IN chimeric HIV-1 with INSTI DRMs had significantly reduced RC compared to the reference wild type strains. Reduced RC exhibited by the viruses was not simply due to poor complementation considering the IN of the subtype A and D in a NL4-3 backbone had slightly higher replication rates than wild type NL4-3. Viruses, UG138-A (N155H; secondary mutations, T97A/L74I,), and UG481-A (Y143R; secondary mutations, T97A/G163R) had lowest RCs at ~30% of NL4-3 and

<30% of the subtype A and D references. UG206-D, with four primary DRMs, E138K, G140A, S147G, and Q148K also had a low RC (29%). However, there was no clear patterns of reduced replication rates based on any of the primary or secondary DRMs (Fig. 25). UG23-A, UG1044-A, and UG35-A replicated at 40 to 45% of the references, while UG119-A, UG537-A/D, UG42-D, and UG11-D were at 50 to 60% of wild type HIV-1s.

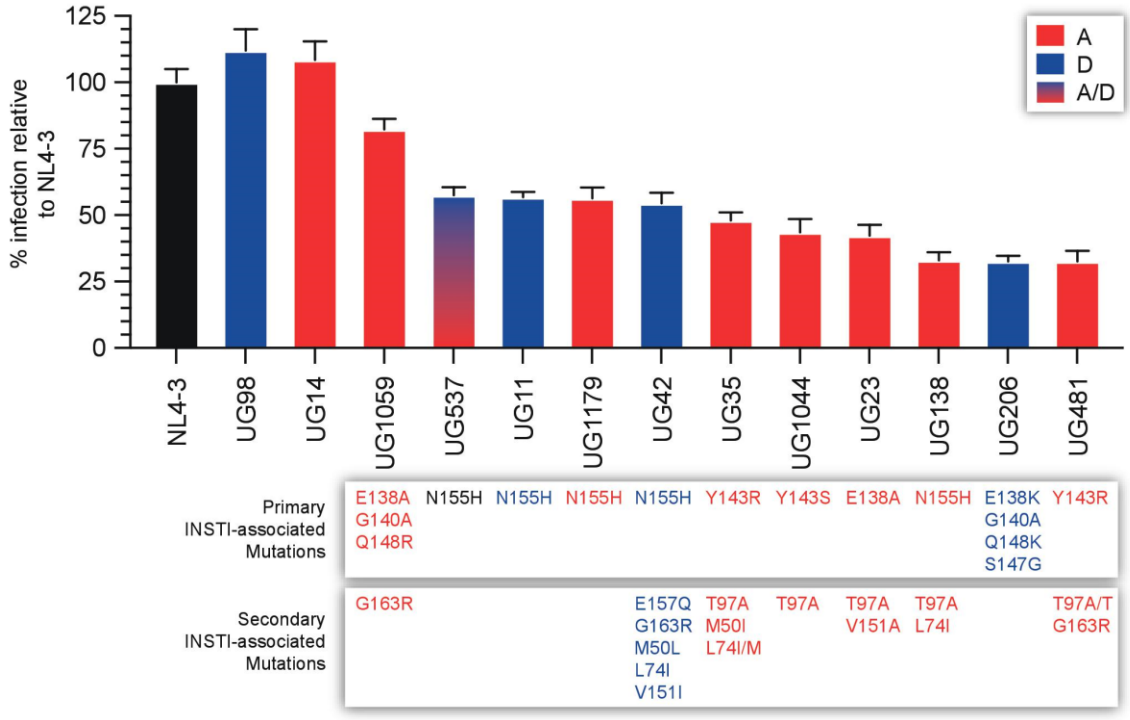

Figure 25. Relative viral infectivity of recombinant viruses. The viral infectivity of resistant mutants compared to controls and wildtype was determined using short-term infection assay in TZM-bl cells. Area under the curve was used to measure the relative decrease in infectivity. The data shown represent means and standard deviation from independent experiments performed in triplicates.

The other chimeric HIV-1 with four primary DRMs in IN (E138A, G140A, Q148R, G163R) had the highest RCs (>80% of wild type).

Cross resistance observed between DTG and elvitegravir

Correlation coefficient of FC values for DTG, raltegravir, and elvitegravir were assessed to determine the level of cross resistance between the INSTIs. DTG FC values for resistance were found to correlate with elvitegravir FC values for resistance (P=0.0073, R=0.8), but not with FC values to raltegravir resistance (P=0.54, R=0.21).

4.6 Discussion

Resistance to INSTIs is well characterized in HIC with a 12, 7 year, and 6-year history of employing raltegravir, elvitegravir, and DTG, respectively. The typical 4 major pathways to INSTIs resistance involve N155H, Q148H/K/R, and Y143R/H/C to raltegravir and elvitegravir, and R263K to DTG and bictegravir as primary INSTI DRMs. Both *in vitro* and *in vivo* selection of DTG resistance is conferred by either an accumulation of INSTI DRMs or the emergence of R263K. Both pathways confers low-level DTG resistance, significant reductions in RC, and often requires the emergence of compensatory mutations, e.g. H51Y with R263K (434).

In this study, we produced 11 chimeric viruses harboring the IN genes of Ugandan patients failing third-line raltegravir-based treatment and with known DRMs. Chimeric IN HIV-1 produced with single DRMs resulted in significant resistance to elvitegravir and raltegravir and 43 to 68% decrease in RC. Even these single DRMs with compensatory/secondary mutations emerging in subtype A and D IN do not confer cross resistance to DTG. HIV-1 containing IN of two patients, UG1059 and UG206 with three and four DRMs (respectively) showed high-level resistance to elvitegravir and raltegravir and over 100-fold cross resistance to DTG. The level of INSTI resistance by these two patient derived viruses are among the highest ever recorded with phenotypic tests. Although the subtype D UG206 had a 70% reduction in RC, the RC for UG1059 was not significantly different from wild type.

Few studies have assessed phenotypic INSTI resistance using the IN derived from INSTI failures in Uganda or in Sub Saharan Africa. One study describes resistance to INSTIs, even DTG in subtype A, B, C, D, F, G, CRF01, CRF02, and other CRFs related to Q148H/K/R resistance pathway but these Q148H/K/R were infrequent in this cohort when compared to INSTI failures in subtype B infected patients (633,640,641). There are, however, several genotypic studies describing common INSTI DRMs in untreated and in INSTI treated patients of Sub-Saharan Africa. For nearly 10 years raltegravir-based treatment has been recommended by WHO for third-line regimens (642) and as such is rarely used in treatment within LIMCs. In just 4 years, generic DTG and TDF/3TC/DTG is now accessible to 3.9 million HIV-1 infected individuals in sub-Saharan Africa and other developing countries (629,631). This important roll out of DTG was briefly interrupted with a report suggesting tetragenic effect in babies born from mothers who start

DTG treatment from the time of conception (643). Nonetheless, there has been no extensive screen for susceptibility of African non-subtype B HIV-1 to DTG and especially not with HIV-1 derived from those failing a raltegravir-based regimen. This study suggests that 5% of raltegravir failures may harbor DTG resistant HIV-1. Even this frequency of DTG cross resistance in raltegravir failures in subtype A and D infected individuals could be a cause of concern. Furthermore, a recent study by our team suggests that 28% of raltegravir failures harbor raltegravir / elvitegravir resistant viruses with previously uncharacterised INSTI DRMs related to subtype A and D (reference submitted for now). Half of these new INSTI-resistant genotypes in subtype A and D HIV-1 displayed cross resistance to DTG.

HIV-1 IN mutation N155H is selected early under raltegravir pressure (644). We have previously shown predominant selection of N155H (17.6%) in HIV-1 patients failing third-line raltegravir - based regimen in Uganda (540), and thus, understanding the susceptibility of viruses with single N155H is important in assessing if DTG could be utilized in a fourth-line regimen given the limited availability of other salvage drugs like enfuvirtide or maraviroc in Uganda. In our study, three viruses with a single N155H mutation were susceptible to DTG (FC, 1.6-2.4) two of these comparable to N155H in subtype B HIV-1 (FC, 1.2) (319). Given the low-level phenotypic resistance associated with DTG failure in subtype B infected individuals, even our modest 2.4-fold decreased susceptibility with N155H to DTG may predict potential treatment failure with DTG-containing ART use as fourth-line treatment in Uganda.

Prolonged exposure to INSTI following treatment failure can lead to an accumulation of primary DRMs and compensatory secondary mutations to increase INSTI resistance and/or restore viral fitness (645). Achieving viral suppression with DTG in patients failing raltegravir or elvitegravir is reduced with each added primary INSTI DRM (G140H/A/S, E138A/K/T) in association with substitutions at Q148, as seen in VIKING-3 study (318). Viral suppression with DTG was also reduced with INSTIs mutations, E138K, G140A, S147G, Q148R and T97A in a subtype C infected, raltegravir-experienced patient from Botswana (646). However, this accumulation of INSTI DRMs is extremely rare in HICs due in part to frequent patient visits, viral load monitoring, and drug resistance testing. In Uganda like many other LIMCs, less frequent clinic visits and viral load testing, intermittent adherence, and limited drug resistant testing could contribute to multi-DRM viruses with high-level INSTI resistance. Two of 18 patients with raltegravir resistance in

this Ugandan cohort had E138A, G140A, Q148R, and G163R in a subtype A virus and E138K, G140A, S147G, Q148K in subtype D. The presence of these four primary INSTI resistance mutations within IN has never been observed in subtype B infected patients, naïve to or failing INSTIs (421). A combination of two primary INSTI DRMs (Q148H, G140S) with secondary mutations, T97A, L74M conferred resistance to DTG in subtype B viruses (647). The loss of RC observed with UG206 could as well be compensated by emergence of secondary mutations to support active infection in presence of drug. Thus, the frequent appearance of multi-DRM HIV-1 in subtype A (as with UG1059) and subtype D (with UG206) upon INSTI treatment failure could have devasting consequences for future roll out of DTG. Hopefully, a 11% frequency of multi-DRM will not persist with larger cohorts on raltegravir-based treatment in sub-Saharan Africa.

Conclusions

In this study, we show that INSTIs resistant viruses in the majority of patients in Uganda failing raltegravir-based third-line treatment remain susceptible to DTG and have impaired RC. However, accumulation of primary INSTI DRMs mutations leads to high level resistance to all INSTIs currently available in LMICs.

Chapter 5

5 High level resistance to bictegravir and cabotegravir in subtype A and D infected HIV-1 patients failing raltegravir with multiple resistance mutations

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5.1 Preface

Accumulation of primary HIV integrase mutations in ART experienced patients infected failing RAL leads to high-level resistance to DTG, RAL, and EVG. However, we have shown previously that DTG remains active against viruses with single primary mutation or combination of secondary mutations. N155H within IN may confer higher resistance to DTG in subtype A and D compared to subtype B viruses. BIC is newly approved second generation INSTI that is becoming available in LMICs, and CAB INSTI is in phase III clinical trial as daily oral and long acting parenteral nanosuspension for monthly or quarterly administration. CAB and BIC have activity against many resistant HIV strains to EVG and RAL and some resistant variants to DTG. Due to wide roll out of INSTIs in LMICs, the number of patients failing INSTIs will also likely increase limiting the available ART options. Therefore, to ascertain possibility of switching ART experienced patients to BIC or CAB, we carried out drug susceptibility assays in-vitro and tested the level of resistance conferred by a diverse group of INSTIs DRMs.

5.2 Abstract

Objectives

The second-generation integrase strand transfer inhibitor (INSTI) bictegravir (BIC) is becoming accessible in low- and middle-income countries (LMICs), and another INSTI (cabotegravir, CAB) has recently been approved as long-acting injectable. Data on BIC and CAB susceptibility in raltegravir (RAL)-experienced HIV-1 subtype A and D infected patients carrying drug resistance mutations (DRMs) remains very scarce in LMICs.

Patients and methods

HIV-1 integrase (IN) recombinant viruses from eight patients failing RAL-based third-line therapy in Uganda were genotypically and phenotypically tested for susceptibility to BIC and CAB. Ability of these viruses to integrate into human genomes was assessed in MT-4 cells.

Results

HIV-1 IN-recombinant viruses harboring single primary mutations (N155H or Y143R/S) or in combination with secondary INSTI mutations, T97A, M50I, L74IM, E157Q, G163R, or V151I were susceptible to both BIC and CAB. However, combinations of primary INSTI-resistance mutations such as E138A/G140A/G163R/Q148R or E138K/G140A/S147G/Q148K led to decreased susceptibility to both CAB (fold change in EC_{50} values from 429 to 1,000x) and BIC (60 to 100x), exhibiting a high degree of cross resistance. However, these same IN-recombinant viruses showed impaired integration capacity (14% to 48%) relative to the wild type HIV-1 NL4-3 strain in the absence of drug.

Conclusions

Though not currently widely accessible in most LMICs, BIC and CAB offer a valid alternative to HIV-infected individuals harboring subtype A and D HIV-1 variants with reduced susceptibility to first generation INSTIs but previous exposure to RAL may reduce efficacy more so with CAB.

5.3 Introduction

HIV-1 drug resistance remains a global threat, even in the era of second-generation HIV-1 strand transfer integrase inhibitors (INSTIs). In low- and middle-income countries (LMICs), the increasing prevalence of HIV-1 drug resistance in the treatment naïve population has required the reduced use of a first-line non-nucleoside reverse transcriptase inhibitors (NNRTI)-based combined antiretroviral therapy (cART), such as efavirenz or nevirapine, to a tenofovir/lamivudine/dolutegravir regimen (629). Nevertheless, prolonged virological failure is common in LMICs due to limited virological monitoring of HIV-infected individuals, leading to accumulation of even INSTI-resistance mutations and reduced susceptibility to dolutegravir (DTG), raltegravir (RAL), and elvitegravir (646,648,649).

Bictegravir (BIC, formally GS-9883) and cabotegravir (CAB, formally S/GSK 1265744 or GSK 744), both structural analogues of DTG, are the latest second generation INSTIs. Two clinical trials in ART-naïve individuals and two trials in virologically suppressed patients (321,457,650) led to approval of BIC in 2018 by FDA as a fixed-dose combination of BIC/FTC/TAF in cART naïve and suppressed patients (<50 copies/ml) with no history of drug resistance. BIC is a potent unboosted once-daily INSTI with a higher *in vitro* barrier to resistance than RAL and EVG, and with limited drug-drug interactions. Its structure has a distinct oxazepane ring attached to a metal chelating scaffold (Fig. 1), which increases flexibility allowing for more adaptability in presence of drug resistance mutations (651). CAB is another analog of DTG from a class of carbamoyl pyridones that has recently been approved by FDA and European Union as long-acting injectable, CAB/rilpivirine for use in HIV patients with undetectable viral loads, stable on current cART, and no drug resistance (652–654). The unique physicochemical and pharmacokinetic properties of the CAB formulation allows its use as a single-daily tablet or long-acting nanosuspension for monthly or quarterly via subcutaneous or intramuscular administration. CAB was recently approved as a fixed-dose long-acting injectable combination of CAB plus the NNRTI rilpivirine after successful clinical trials in HIV infected individuals (324,655).

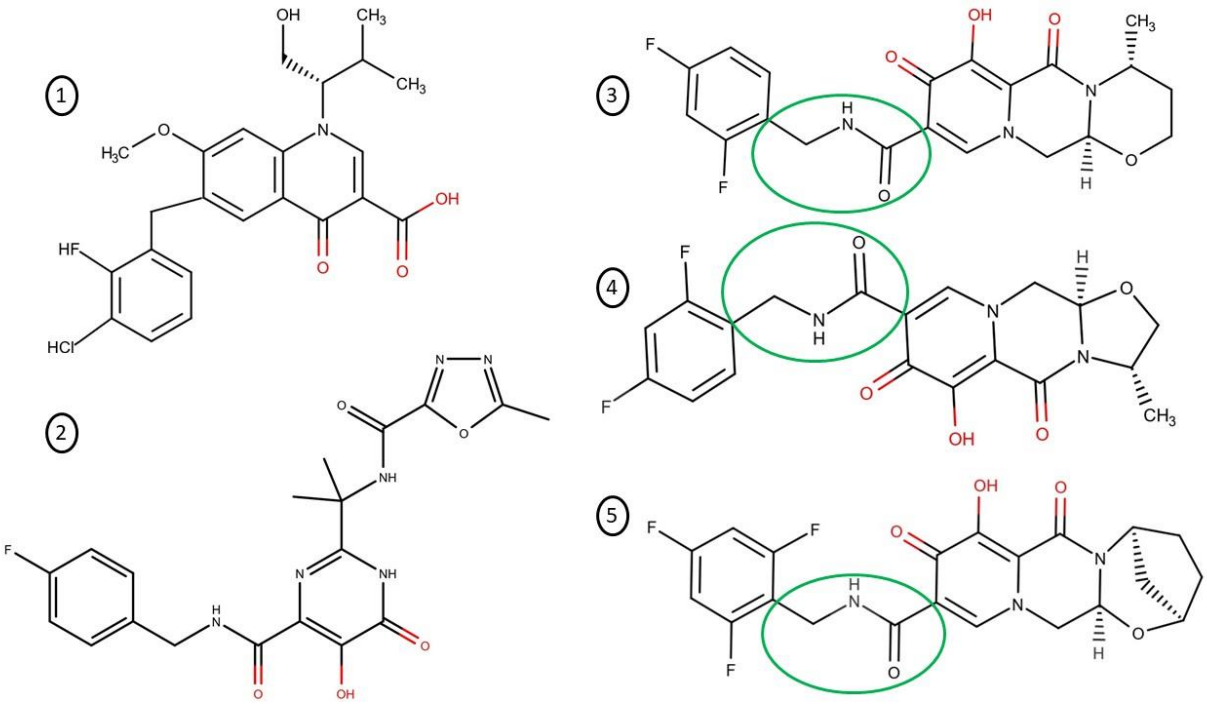

Figure 26. The chemical structures of different INSTIs. 1) Elvitegravir, 2) Raltegravir, 3) dolutegravir, 4) cabotegravir, and 5) bictegravir. The coplanar oxygen atoms are highlighted in red and green circle highlights extended linker a common feature to all second generation INSTIs.

BIC has shown broad activity *in vitro* as an INSTI against recombinant viruses and clinical isolates carrying primary mutations associated with resistance to RAL and elvitegravir (459,656). However, mutations at position Q148 of the HIV-1 integrase (IN) confer a wide range of resistance to BIC *in vitro* when combined with other amino acid substitutions (657). Selection of the Q148R mutation was observed in two patients on either oral or long injectable CAB in the LATTE clinical trials (655,658); however, CAB has shown activity against viruses harboring T66I, Y143R, N155H, E92Q, Q148H/K/R and G140S/Q148H/K/R (<7-fold) (463).

By mid-2020, transition to generic DTG-based first line had been implemented in 100 LMICs, however, the distribution of DTG at large scale in LMICs has coincided with the emergence of SARS-CoV-2, responsible for the COVID-19 pandemic (659,660). Unfortunately, this may lead to increasing emergence of INSTI-resistance mutations associated with poor therapy adherence due to reduced access to pharmacies and healthcare providers, or disruptions in the distribution of antiretroviral drugs (661). Currently, patients failing third-line RAL-based regimens can only be

switched to optimized dosage of DTG. Twice-daily DTG dosage of 50mg or 100mg may rescue viral suppression in patients carrying resistance mutations at residue G140 and Q148 of IN (318,662). However, some cART experienced patients infected with subtype B or non B HIV-1 strains with multiple DRMs are already resistant to DTG (646,648,649,657). With prevailing challenge of access to more potent antiretrovirals options in LMICs, it becomes very crucial to assess susceptibility of viruses harboring diverse INSTI-associated mutations to BIC and CAB (663).

In this study, non-B IN recombinant viruses derived from eight Ugandan patients failing RAL-containing regimens and carrying single or multiple primary INSTIs mutations with or without secondary INSTIs mutations, were phenotypically tested for susceptibility to CAB and BIC. Although patient-derived IN-recombinant viruses carrying single primary INSTI mutations (or in combination with secondary INSTI mutations) were susceptible to BIC and CAB, viruses harboring multiple primary INSTI-resistance mutations (i.e., E138A/G140A/G163R/Q148R and E138K/G140A/S147G/Q148K) led to reduced susceptibility to both novel INSTIs.

5.4 Methods

Clinical samples

The study patients n=8 failing RAL-based third-line therapy with INSTIs-resistance mutations were part of a cohort of n=51 HIV-1 infected patients failing RAL-based third line at the Joint Clinical Research Center (JCRC) in Kampala, Uganda as described previously (540,649). JCRC was the first center to provide generic cART in Uganda starting in early 2000 (531,533) and has since provided HIV-1 treatment to over 200,000 HIV-1 patients in Uganda and other African countries since 1990. Samples of patients with virological failure detected during routine checkups are sent to Case Western Reserve University -Center for AIDS Research (CFAR) laboratories at JCRC for sanger HIV-1 genotyping tests (488). The CFAR laboratory is a World Health Organization (WHO), College of American Pathologist (CAP), and National Institutes of Health-virology quality assurance (NIH-VQA)-accredited laboratory. HIV-1 patients who provided written consent and were experiencing virological or immunological failure described as plasma HIV-1 RNA load >1,000 copies/ml and/or $CD4^+$ T cell counts below 250 cells/mm^3 were included in the study. Ethical clearance was obtained from the IRBs at the JCRC and University Hospitals Cleveland Medical Center/Case Western Reserve University (EM-10-07 and 10-05-35).

RNA extraction and PCR amplification

Plasma samples from all eight patients were used to extract HIV-1 viral RNA (QIAamp viral RNA Mini Kit, Qiagen) and the IN-coding region was amplified using a Superscript III single RT-PCR system (Thermo Fisher Scientific) as previously described (540).

Sanger sequencing analysis

Purified and quantified IN amplicons were cycle sequenced using Big Dye Terminator v3.1 (Thermo Fisher scientific) and analyzed as previously described (540). Briefly, PCR products were sequenced on an ABI 3730XL to cover the full length of the IN (288 amino acids). Raw sequences were exported and analyzed in RECall (beta v3.02) program as recommended by the WHO (606). To check for presence of DRMs, analyzed sequences were exported to Stanford HIV-1db Sierra web service algorithm v8.3 (637).

Library preparation and deep sequencing

To confirm the presence of drug resistance mutations originally identified by Sanger sequencing, an amplicon-based deep sequencing method was used as previously described (540). Briefly, two overlapping PCR products spanning full-length of INT were purified using (Agencourt AMPure XP; Beckman Coulter) and quantified using (Quant-iT Picogreen dsDNA assay kit; Thermo scientific). The barcodes were added using Nextera XT index Kit v2 (Illumina) and paired-end sequencing done on a MiSeq instrument (Illumina). Analysis was performed with MiSeq Reporter analysis software version 2.6 (Illumina) and drug resistance interpretation was done using the Stanford HIV-1db Sierra web service algorithm v8.3 (637).

Cells and antiviral compounds

TZM-bl, U87.CD4.CXR4, MT4, and HEK293T cell lines were obtained through the AIDS Research and Reference Reagent Program, division of AIDS, NIAID, NIH. TZM-bl cells, and HEK293T were maintained in DMEM supplemented with 10% FBS, 100 μg/ml penicillin-streptomycin. U87.CD4.CXR4 cells were maintained in DMEM supplemented with 10% FBS, 100 μg/ml penicillin-streptomycin, 300 μg/ml G418, and 1 μg/ml puromycin (Invitrogen, Carlsbad, CA). MT4 cells were maintained in RPMI supplemented with 10% FBS. All cell lines were sub-cultured every 3 to 4 days at 37°C under 5% CO_2. CAB and BIC were purchased from Selleck chemicals (Houston, TX).

Construction of HIV-1 integrase recombinant viruses

Full-length IN- PCR products were recombined into near full-length HIV-1 (pREC_NFL_IN/URA3) vector using transfected *Saccharomyces cerevisiae* MYA-906 cells (ATCC) based on the yeast homologous recombination-gap repair system as previously described (638). Briefly, plasmids were extracted from the yeast cells using phenol/chloroform (Thermo Fisher scienfific) and transformed into electrocompetent *Escherichia coli* Stbl4 cells (Thermo Fisher scienfific). Plasmids were then extracted from the bacteria using Qiagen Miniprep kit (Hilden, Germany). The presence of mutation(s) in the generated plasmids was confirmed by Sanger sequencing. pREC_NFL_IN plasmids were co-transfected into HEK293T cells (3×10^4 cells/well) along with the complementing plasmid pCMV_cplt using Fugene 6 reagent (Promega,

Madison, WI) (638). Produced heterodiploid virus particles containing one copy of the pREC_NFL_IN and cplt HIV-1 RNAs were further propagated on U87.CD4.CXCR4 cells to produce a complete HIV-1 genome. Following virus propagation, HIV-1 RNA was RT-PCR amplified and Sanger sequenced to confirm the presence of the different DRMs.

Virus titration

The titers of the IN-recombinant viruses were measured using short-term infectivity assays in TZM-bl cells using Galacto-Star chemiluminescent reporter gene assay (Thermo Fisher Scientific), which measures expression of the β galactosidase enzyme under HIV-1 Tat expression. Briefly, 20,000 TZM-bl cells were seeded in the presence of polybrene (1mg/ml) to each well of 96-well plate. The cells were infected with 3-fold serially diluted virus and cultured in 37°C and 5% CO_2 incubator for 48 hrs. The cells were washed with 200µl of 1X PBS and lysed with 10µl of lysis buffer for 10 mins. To the cell lysate, 100µl of reaction buffer and Galacton-star substrate was added and incubated for 1 hr at room temperature. The expression of β-galactocidase enzyme was measured on Cytation 5 plate reader (BioTek USA).

INSTI susceptibility assay

The susceptibility of patient-derived IN-recombinant viruses to BIC and CAB was determined using short-term resistance assays based on TZM-bl cells. Briefly, 20,000 cells were seeded to each well of 96-well plate and infected with the IN-recombinant viruses, or three control HIV-1 strains (i.e., subtype B NL4-3, subtype A UG14, and subtype D UG98) in the presence of 10-fold dilutions of BIC and CAB (100 µM to 10^{-8} µM) and polybrene (1mg/ml), at an MOI of 0.05 IU/ml in quadruplicate. After 48 hr incubation at 37°C and 5% CO_2, virus replication was quantified using Galacto-Star chemiluminescent reporter gene assay as described above. Drug dose response curves were generated using nonlinear regression curve fitting features of GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA). Drug resistance was expressed as fold change (FC) in effective concentration 50 (EC_{50}) between HIV-1 controls and IN-recombinant viruses.

HIV-1 integration assay

The relative HIV-1 integration into cellular DNA was determined by carrying out Alu-gag qPCR as previously described (428),(664), with some modifications. Briefly, 30,000 MT-4 cells were infected with normalized viruses (MOI of 0.05 IU/ml) in the presence of 1mg/ml polybrene.

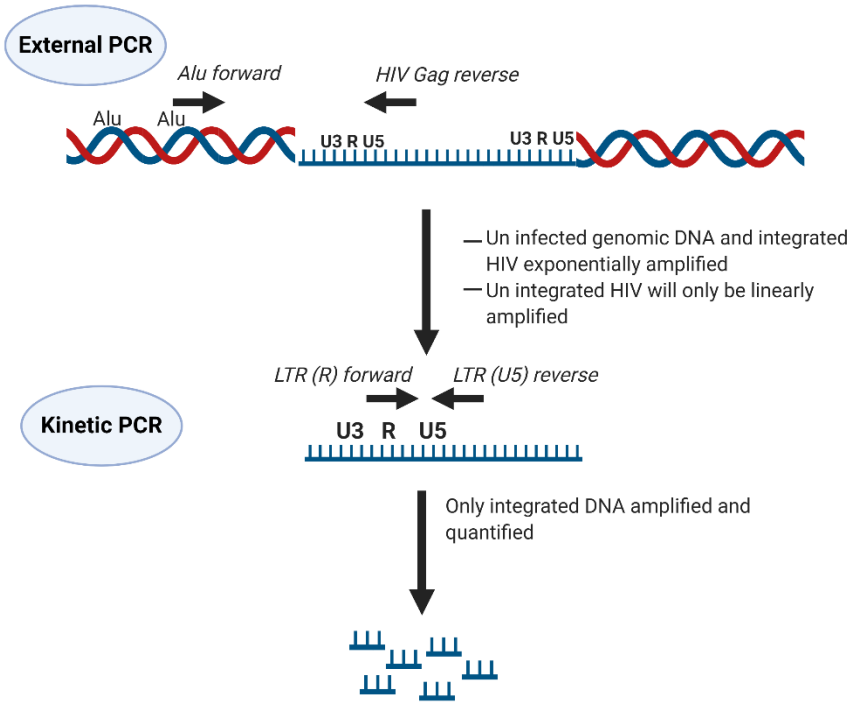

Figure 27. The Alu-Gag PCR workflow. In the first round of PCR, primer Alu forward aligns to the alu repeats which exist randomly in human genome. The reverse primer, Gag reverse aligns at U5 of 5′ end of HIV genome. In the second round of PCR, LTR (R) forward primer aligns at R and reverse primer LTR (U5) at U5 of 5′ end of HIV genome to produce 129bp product that is quantified by qPCR machine.

The HIV-1 protease inhibitor darunavir (1µM) was added to allow one round of infection and cells were cultured in 37°C and 5% CO_2 incubator for 72 hours. Genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) and normalized using β-globin gene using primers described previously. The DNA was amplified using Alu-Gag PCR with first round PCR primers, Alu Forward (5′ GCC TCC CAA ACT GCT GGGATT ACA G-3′) and HIV-1 Gag reverse (5′

GTT CCT GCT ATG TCA CTT CC-3′) and kinetic PCR primers, LTR (R) forward, (5′-TTA AGC CTC AATAAA GCT TGC C-3′), LTR (U5) reverse, (5′-GTT CGG GCG CCA CTG CTA GA-3′) and probe (5′-FAM-CCA GAG TCA CACAAC AGA GGG GCA CA-TAMRA-3′) previously described (664). The first round PCR was done using platinum taq DNA polymerase High fidelity (ThermoFisher scientific) and reaction mix consisting of 10X High fidelity PCR buffer, 50mM Mgcl2, 10mM dNTP mix, 20µM of each primer and water. The PCR gram was done as follows: initial denaturation at 94°C for 2 mins, denaturation at 94°C for 15 secs, annealing at 55°C for 15 secs, extension at 68°C for 3 mins and final extension at 68°C for 7 mins. The kinetic PCR was done using TaqMan Fast advanced master mix (ThermoFisher scientific), 20µM of each primer and probe, water, and template. QuantStudio real time system (ThermoFisher scientific) was used under the following conditions, UNG incubation at 50°C for 2 mins, polymerase activation at 95°C for 10 mins, denaturation at 95°C for 15 secs and annealing/extension at 60°C for 1 min. The relative HIV-1 integration was quantified using a standard curve generated by dilution series of DNA from infected MT-4 cells (dilution using DNA from uninfected cells).

Global occurrence of Q148H/K/R mutations

To assess global occurrence of Q148H/K/R mutations and associated INSTIs primary resistance mutations in patients failing RAL, we analysed all the available HIV-1 INSTI-associated mutations from the Drug Resistance HIV Stanford Database (https://hivdb.stanford.edu/). All sequence data with original reference, patient identifier, isolate name, accession number, and treatment history was retrieved for the analysis. The search was conducted on August 28, 2020 and search terms used were, “integrase, raltegravir, HIV-1 and subtypes.”

Statistical analyses

Statistical analyses were performed using non-linear regression in GraphPad Prism 8.1.2 (GraphPad Software, La Jolla, CA). The level of cross resistance between INSTIs was analyzed using Spearman’s rank order test. The means of EC_{50} for BIC, CAB, DTG, RAL, and elvitegravir, were compared using one-way ANOVA and p-values <0.05 were considered statistically significant.

5.5 Results

Individuals failing ART with INSTI-associated mutations

We previously described a cohort of sixty HIV-1-infected patients failing RAL-based third-line regimen in Uganda (540). From this group of individuals, we identified eight patients carrying HIV-1 strains with a variety of mutations associated with resistance to INSTIs (the most common being N155H), as well as two patients infected with HIV-1 strains lacking INSTI-associated mutations (Table 9). The patients median age was 30.5 years [Interquartile range (IQR), 26.25-36.25 years] and the median plasma HIV-1 RNA load was 85,091 copies/ml with (IQR, 3687.5-570,480 copies/ml). As expected, most patients were infected with subtype A HIV-1 strains (62.5%), followed by subtype D (25%) and recombinant A/D (12.5%). Five of the eight patients were treated with RAL/LPV/RTV, while the other three individuals were RAL/DRV/RTV, RAL/TDF/3TC/LPV/RTV, and RAL/TDF/FTC/DRV/RTV-experienced (Table 9).

Table 9. The clinical characteristics of study patients

Patient ID	Subtypes	Age	Viral load (copies/ml)	Regimen	Resistance mutations
UG23	A	51	850	LPVr/RAL	**E138A**, T97A, V151A
UG35	A	31	2,293,840	DRVr/RAL	**Y143R**, T97A, M50I, L74IM
UG42	D	23	155,982	LPVr/RAL	**N155H**, E157Q, G163R, M50L, L74I, V151I
UG206	D	ND	1,515,000	LPVr/RAL	**E138K, G140A, Q148K, S147G**
UG1059	A	ND	14,200	TDF/3TC/LPVr/RAL	**E138A, G140A, Q148R**, G163R
UG481	A	38	3,914	TDF/FTC/RAL/DRVr	**Y143R**, TA97AT, G163R
UG537	A/D	ND	255,641	LPVr/RAL	**N155H**
UG1179	A	30	ND	LPVr/RAL	**N155H**
UG11	D	25	ND	ATVr/RAL	**N155H**
UG14	A	ND	3,008	ART naive	None
UG98	D	ND	ND	ART naive	None

The HIV-1 subtype was predicted using SCUEAL subtype classification algorithm. Viral loads were assayed using Abbott m2000sp/rt or Roche COBAS Amplicor Monitor ultrasensitive tests, v1.5. In bold, major INSTIs resistance mutations. Abbreviations: cART, combined antiretroviral therapy; ND, not determined; INSTI, integrase strand transfer inhibitor; DRVr, darunavir/ritonavir; LPVr, lopinavir/ritonavir; RAL, raltergravir; 3TC, lamivudine; TDF, tenofovir; FTC, emitricabine

BIC and CAB retain activity in presence of single N155H or Y143R and added secondary mutations.

In this study, IN recombinant viruses carrying diverse INSTIs-associated resistance mutations from patient-derived IN (n=8) were tested for their susceptibility to BIC and CAB (Table 9). The RAL and elvitegravir -resistant mutant N155H remained susceptible to both BIC (1-2.3-fold) and CAB (1.3-6.3-fold), and emergence of secondary mutations, E157Q, G163R, M50L, L74I, and

V151I in context of N155H, did not affect susceptibility to BIC (0.8-fold) and CAB (1.3-fold) respectively (Table 10, Fig.28, and Fig.29). Viruses carrying the primary mutation to RAL, Y143R in combination with secondary mutations T97AT, G163R, M50I, and L74IM remained susceptible to BIC (FC, 1.2-1.3) and CAB (1.7-2.6).

Table 10. The mean EC_{50} and fold-change in EC_{50} of recombinant viruses

	BIC			CAB		
Patient ID	EC50	95% CI for EC50	FC in EC50	EC50	95% CI for EC50	FC in EC50
nfl4-3	2.78	1.124-1.919	1	1.36	2.336-3.325	1
UG14	3.8	3.057-4.761	1.4	1.38	1.137-1.675	1
UG98	5.0	3.934-6.385	1.8	1.87	1.577-2.233	1.3
UG481	3.4	1.79-3.261	1.2	2.4	2.533-4.616	1.76
UG537	2.7	1.393-2.416	1	1.8	2.277-3.292	1.3
UG23	2.4	1.672-3.604	0.8	4.7	2.985-7.624	3.3
UG42	2.5	2.151-3.126	0.8	1.8	1.467-2.299	1.3
UG35	3.7	2.756-5.081	1.3	3.6	2.515-5.367	2.6
UG1179	3.9	2.823-5.368	1.4	2.8	1.909-4.346	2.0
UG11	6.6	4.856-9.173	2.3	8.7	5.64-13.83	6.3
UG1059	166.1	495.3-686.9	59.7	584.1	126.3-217.5	429.1
UG206	369.1	2015-3493	132.7	2650	319.5-426.2	1948.5

The EC_{50} and 95% CI for EC_{50} was determined using nonlinear regression analysis in GraphPad prism. The nfl4-3 was used as wild type in the assays. Abbreviations: BIC, bictegravir; CAB, cabotegravir; EC_{50}, 50% effective concentration; CI, confidence interval. The fold-change values are relative to NL4-3 wild type.

The mutation E138A selected by RAL, elvitegravir, and DTG did not reduce susceptibility to either BIC (0.8-fold) or CAB (3.3-fold) in presence of secondary mutations T97A and V151A (Table 10, Fig.28, and Fig.29).

Combination of multiple primary INSTI-associated mutations leads to high-level resistance to both BIC and CAB.

Prolonged virological failure on same HIV-1 regimen can result in accumulation of HIV-1 drug resistance mutations in a patient viral population (665). Severe reduced susceptibility to DTG (>100-fold) was previously shown with IN-recombinant virus UG1059 carrying the primary E138A, G140A, Q148R mutations as well as the secondary mutation G163R (649).

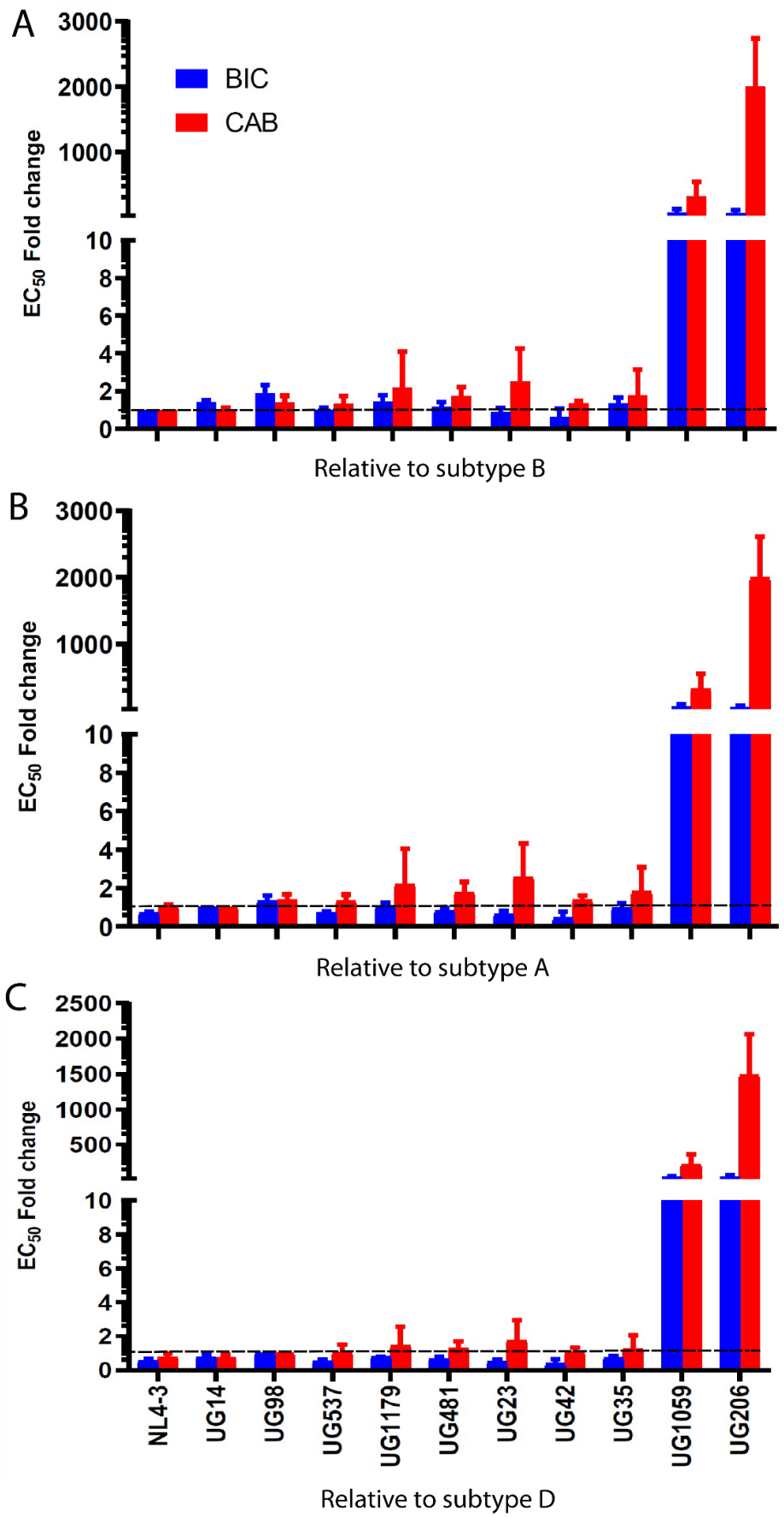
A
BIC
CAB
EC50 Fold change
3000
2000
1000
10
8
6
4
2
0
Relative to subtype B
B
EC50 Fold change
3000
2000
1000
10
8
6
4
2
0
Relative to subtype A
C
EC50 Fold change
2500
2000
1500
1000
500
10
8
6
4
2
0
NL4-3
UG14
UG98
UG537
UG1179
UG481
UG23
UG42
UG35
UG1059
UG206
Relative to subtype D

Figure 28. The EC_{50} (nM) and fold-change in EC_{50} of recombinant viruses by subtype. The susceptibility of IN-recombinant viruses, UG537 (with mutation N155H), UG1179 (N155H), UG481 (Y143R, T97AT, G163R), UG23 (E138A, T97A, V151A), UG42 (N155H, E157Q, G163R, M50L, L74I, V151I), UG35 (Y143R, T97A, M50I, L74IM), UG1059 (E138A, G140A, Q148R, G163R), and UG206 (E138K, G140A, Q148K, S147G) to BIC and CAB was determined using TZM-bl cells. The mean EC_{50} (nM) values from independent experiments run in quadruplicates were used to determine fold-change in EC_{50} (nM) of recombinant viruses harboring INSTIs-resistance mutations relative to subtype B, A, and D references (panels A, B, and C). The error bars represent ± SD in EC_{50} values between replicates of independent experiments. The horizontal line represents fold-change of 1.

In context of BIC and CAB, UG1059 showed a decreased susceptibility to BIC (60-fold), and >100-fold to CAB.

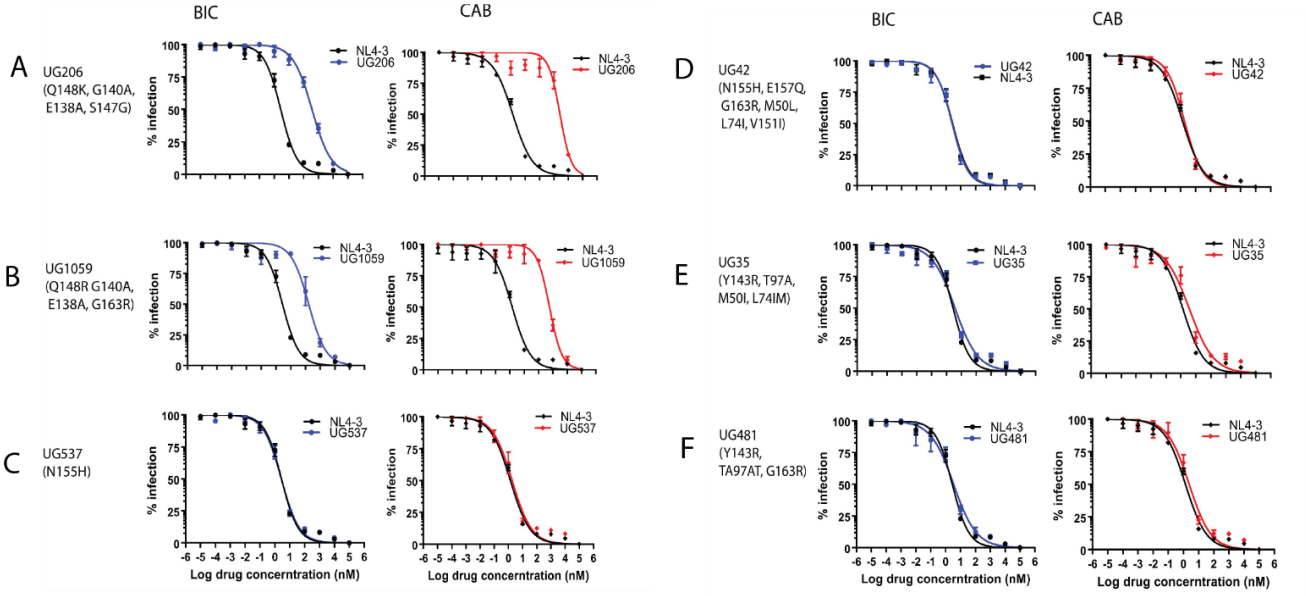

Figure 29. The susceptibility of recombinant viruses to CAB and BIC. The susceptibility of recombinant viruses to INSTIs, BIC and CAB was determined using short-term infectivity assay in TZM-bl cells. Each experiment was done in quadruplicates. The change in EC_{50} (nM) of recombinant viruses harboring INSTIs- resistance mutations was determined in reference to NL4-3 wild type. Panel A- UG206, B- UG1059, C- UG537, D- UG42, E- UG35, and F- UG481, drug susceptibility to BIC (left panel) and CAB (right panel) respectively.

Interestingly, the subtype D virus UG206 also resistant to DTG, carrying E138K, G140A, Q148K, and S147G mutations, was even more resistant to BIC and CAB (>100-fold and >1,000-fold)

respectively (Table 10, Fig.28, Fig.29, and Fig.30). The NL4-3 alone or subtype B HIV-1 carrying a wild type subtype A or D IN coding region showed no difference in susceptibility to BIC and DTG with EC_{50} values previously reported for wild type HIV-1. (458,463) Overall, BIC showed more potency against viruses carrying various INSTIs-associated resistant mutations compared to CAB (p=0.03). IN-recombinant viruses carrying multiple primary INSTIs- resistance mutations showed substantial reduced susceptibility to both BIC and CAB but EC_{50} values for BIC were significantly lower compared to CAB for both UG206 (p=0.003) and UG1059 (p=0.0016; Fig. 29 A-B).

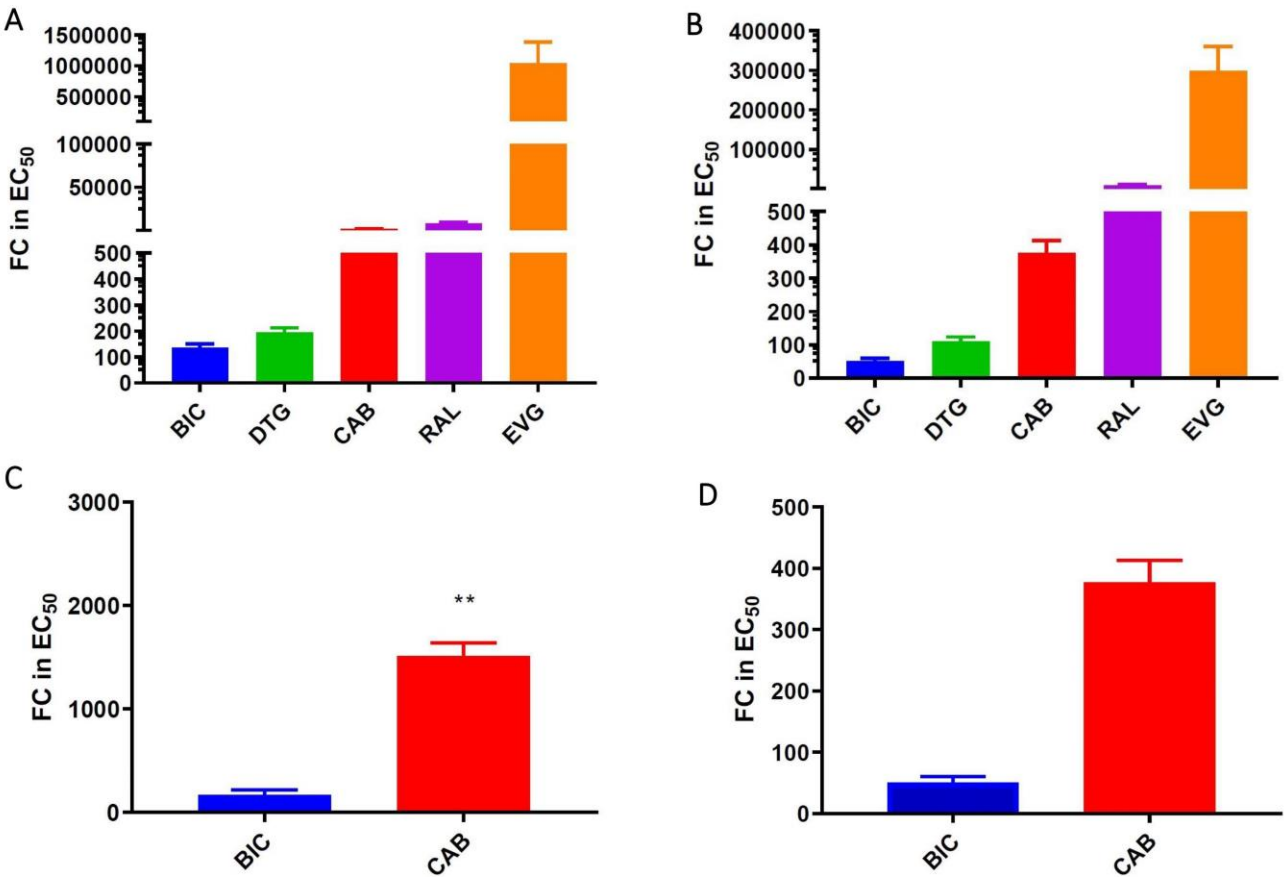

Figure 30. The fold-change (FC) in EC_{50} of recombinant viruses. A) the FC in EC_{50} (nM) of recombinant viruses UG206 relative to wild type NL4-3 for BIC, CAB, DTG, RAL, and EVG. B) the FC in EC_{50} of recombinant virus UG1059 relative to wild type NL4-3 for BIC, CAB, DTG, RAL, and EVG. C) the FC in EC_{50} (nM) of BIC and CAB for recombinant virus UG206, D) UG1059. The FC in EC_{50} (nM) of recombinant viruses harboring INSTIs-resistance mutations relative to wild type NL4-3 were determined in short infection assay in TZM-bl cells. The mean

EC_{50} of CAB and BIC were compared using nonparametric two-tailed t-test, p=0.005 was considered statistically significant.

Both BIC and CAB are second generation INSTIs targeting IN gene for inhibition of HIV-1 replication. To determine degree of cross resistance between BIC and CAB for different viral genotypes tested, log FC in EC_{50} values for BIC resistance were correlated with log FC in EC_{50} values for CAB resistance. Significant cross resistance was observed as shown by strong correlation coefficient, r= 0.995, p= 0.0001, and slope, 0.69 between BIC and CAB (Fig. 30).

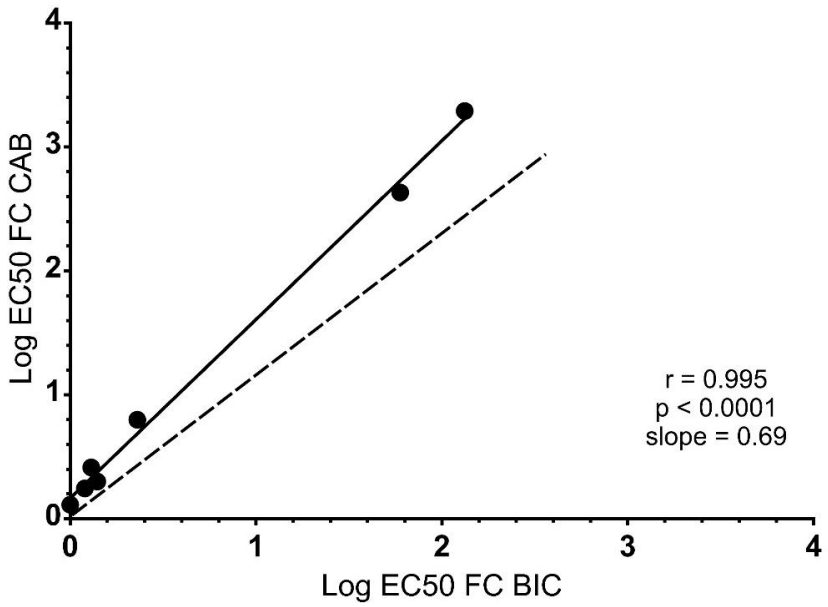

Figure 31. The correlation of fold change values in EC_{50} between BIC and CAB for the same patient sample. The correlation of resistance between BIC and CAB was determined by spear man's correlation coefficient. The fold change (FC) values are relative to NL4-3 wild type.

IN-recombinant viruses carrying single or multiple primary INSTIs-resistance mutations exhibit impaired integration capacity into cellular DNA.

HIV-1 integration into host chromatin strongly favors active transcription sites (231). To test impact INSTIs-resistance mutations may have on integration, we quantified the relative amounts of viral integration using a quantitative Alu-gag qPCR (Fig. 32) as described previously. (664) Viruses UG35 and UG42 carrying the primary N155H or Y143R mutations with additional secondary mutations showed only 25%-29% of the capacity to integrate into the host genome compared to wild type NL4-3 or NL4-3 carrying the wild type subtype A or D IN. Presence of a single N155H mutation in the UG537 or UG1179 viruses led to a 50% reduction in integration. The UG1059 virus carrying mutations E138A, G140A, Q148R, and secondary G163R had the lowest capacity of integration with only 14% compared to wild type NL4-3 (Fig. 32).

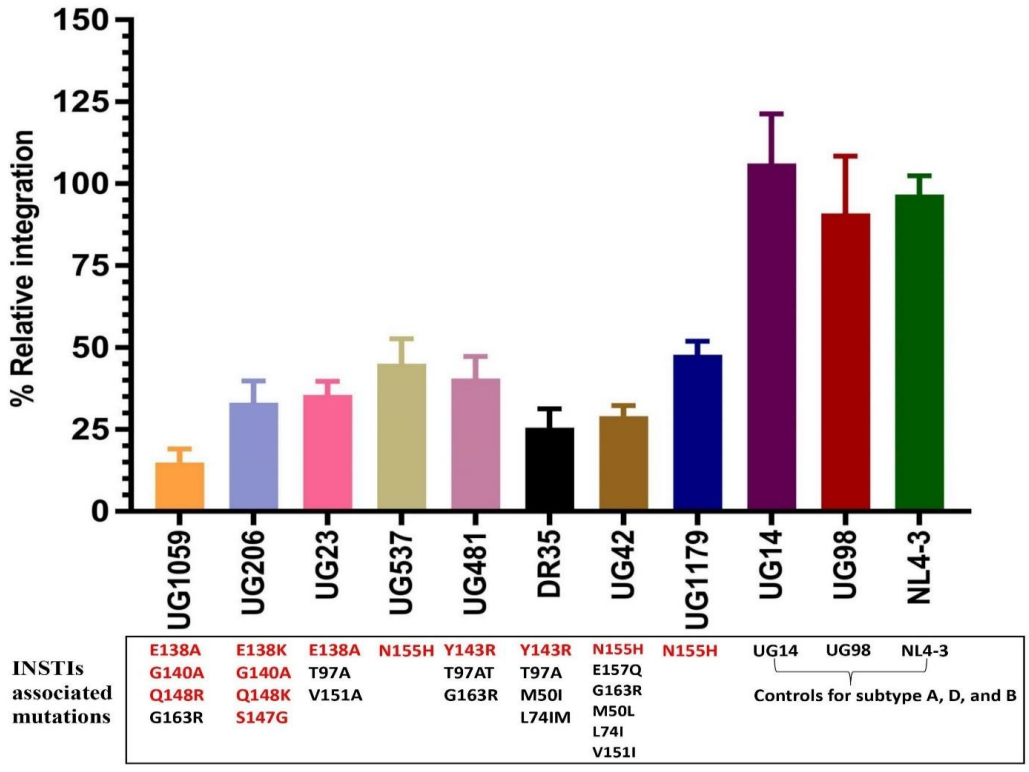

Figure 32. The relative integration capacity of recombinant viruses harboring diverse INSTIs associated DRMs. The ability of recombinant viruses to integrate into host chromatin was determined in MT-4 cells using Alu-gag assay and percentage of integration by mutant relative to NL4-3 was determined.

Prevalence of Q148HRK mutations in RAL failing patients infected with subtype B and non-B HIV-1 isolates

Viruses, UG1059 and UG206 with high-level resistance to both BIC and CAB carry Q148K/R with additional E138A, G140A, G163R and E138K, G140A, and S147G INSTI-associated mutations, respectively (Table 10, Figs.28, Fig.29, and Fig.30). However, this combination of INSTI resistance mutations have not be previously reported which prompted us to assess global prevalence of Q148HRK and other mutations in subtype B and non-subtype B infected individuals failing RAL (Table 11, Fig 33). We used Stanford HIV database (https://hivdb.stanford.edu/) to analyse HIV-1 INSTIs-resistance mutations from HIV-1 infected patients failing on RAL (n=1,653).

Table 11. Prevalence of Q148HKR and associated primary mutations in patients failing on raltegravir.

	Subtype B Total isolates=1442	Subtype Non-B[a] Total isolates=211
Mutation	n (%)	n (%)
Q148QHR, E138EKA, G140GSA	7(0.49)	0 (0.0)
Q148QRH, E138EKA	11(0.76)	2(0.95)
Q148QR, N155NH, E138EK	2(0.14)	0 (0.0)
Q148QHR, N155NH, G140GS	10(0.69)	0 (0.0)
Q148H, G140GS, Y143YCHR	5(0.35)	0 (0.0)
Q148R, R263RK, G140S	2(0.14)	0 (0.0)
Q148QR, N155NH	4(0.28)	0 (0.0)
Q148QRH	15(1.04)	2(0.95)
Q148QHRK, G140GSCA,	281(19.49)	4(1.9)
Q148HQRK, E138EAKT, G140SGCA	54(3.74)	2(0.95)
Q148RQ, E138KE, S147GS	6(0.42)	0 (0.0)
Q148QKR, N155NH, E92EQ, G140GS	7(0.49)	0 (0.0)
Q148H, E138EA, G140SG, Y143H	3(0.21)	0 (0.0)
Q148QRK, N155NH, E138EK, G140GA	3(0.21)	0 (0.0)
Q148KR, E138K, G140GS, S147SG	1(0.07)	1(0.47)
Others[b]	8(0.55)	0 (0.0)

INSTIs associated resistance mutations in patients failing on RAL containing regimens were retrieved from Stanford HIV drug resistance database (https://hivdb.stanford.edu/). [a] HIV-1 subtype A, C, D, F, G, H, J, K, CRF01_AE, CRF02_AG, CRF12_BF, CRF05_DF, CRF47_BF, CRF06_cpx, CRF11_cpx, CRF09_cpx. [b] Q148HKR and associated primary mutations observed once in patient database: S147G, Q148R; T66TK, G140GS, Q148QR; E138EK, S147G, Q148R; E138K, Y143YCHR, Q148QR; E138EA, S147SG, Q148QR, N155NH; G140S, Q148H, V151VA; G140GS, Y143YR, Q148QH, N155NH; and E92EQ, G140GS, Y143YR, Q148QR, N155NH.

HIV-1 subtype B comprised the majority of sequences carrying Q148HRK (87%, 1442/1653) while non-B HIV-1 subtypes represented 13% of sequences (211/1653). The single Q148HRK or in combination with other primary mutations was found in 29% (419/1442) of HIV-1 subtype B

but only 5.2% (11/211) of non-B subtypes infected patients failing RAL-based treatments. The Q148HRK, G140SCA combination was most dominant in HIV-1 subtype B (19.5%) and non-B subtypes (4.9%), with Q148HRK, E138AKT, G140SCA combination occurring in (3.7%) of subtype B and (1%) of non-B HIV-1 subtypes infected patients. The combination of Q148HRK and N155H are uncommon in all HIV-1 derived INs from patients failing RAL (1.8%) regardless of subtypes (Table 11, Fig 33).

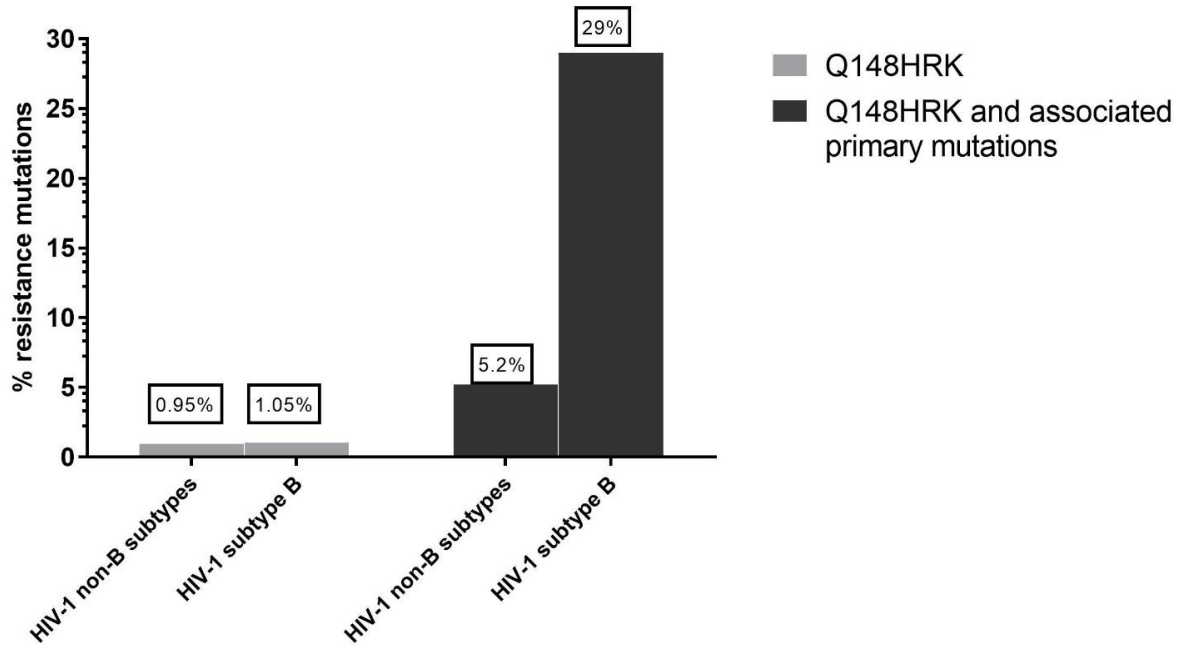

Figure 33. The prevalence of Q148HRK and associated primary INSTIs-resistance mutations in HIV-1 subtype B and non-B subtype viruses in patients failing RAL-based regimens. The number of HIV-1 infected patients failing on RAL with single Q148HKR with or without other primary INSTIs mutations was assessed from HIV drug resistance database by Stanford University (https://hivdb.stanford.edu/).

Our detection of these multiple INSTI resistance mutations in two HIV-1 infected individuals would appear to be a chance and rare discovery. However, it is important to point out that our IN sequences represents 51 of 71 of subtype A and D sequences in the Stanford HIV database and 51 of 211 of all non-subtype B sequences from individuals failing any INSTI-based treatment.

5.6 Discussion

Increased prevalence of drug resistance in the treatment naïve HIV infected population of many LMICs has led to switch from the administration of two NRTIs + an NNRTI (efavirenz or nevirapine) to the TDF/3TC/DTG regimen for individuals initiating cART. Current WHO guidelines also recommend switch of patients on first line with no history of INSTI treatment to TDF/3TC (or FTC)/DTG (666). Despite wide roll out of DTG in LMICs, there is increasing case-reports of DTG failure in patients started on DTG-based regimens, and in INSTIs experienced patients carrying mutations including Q148HKR (318,649).

The recently approved second generation INSTI BIC is available as a part of single-fixed dose formulation of TAF/FTC/BIC (Biktarvy). This TAF/FTC/BIC treatment is indicated in ART naïve and virologically suppressed patients (viral load <50 copies/ml) with no history of treatment failure and drug resistance (667). As previously reported with DTG (308,319,649), BIC also shows potent inhibition of a broad range of drug resistant viruses *in vitro*. (668,669) Before recent CAB approval as long-acting injectable, CAB was tested in combination with rilpivirine in both ART naïve and virologically suppressed patients as oral or long acting injectable in the LATTE 1, LATTE 2, ATLAS and FLAIR clinical trials. In LATTE 1 and 2, INSTIs associated Q148R mutation emerged while in the FLAIR study, G140R, Q148R, E138A/T/K, and ATLAS, N155H mutation were observed in patients with virological failure (289,655,658,670).

In this study, we tested the HIV inhibition profiles of CAB and BIC using virus carrying patient-derived subtype A and D INs. Recombinant viruses carrying either single primary INSTI resistance mutations (N155H, Y143R, E138A) remained susceptible to both BIC and CAB. We have previously shown that N155H as a predominant INSTIs-resistance mutation (17.6%) in HIV-1 patients failing third-line RAL-based regimen in Uganda (540). Viruses carrying N155H in subtype A or D IN were susceptible to BIC (1-2.3-fold), comparable to that observed with HIV-1 subtype B virus (1-fold) (668). In HIV-1 infected patient failing RAL, Q148HRK mutation emerges later in the course of infection replacing N155H mutation of higher replication fitness but exhibiting lower resistance to RAL (671). In our study, subtype A recombinant virus carrying E138A, G140A, Q148R, and G163R mutations and subtype D virus carrying E138K, G140A, Q148K, and S147G mutations showed high level resistance to both CAB and BIC. In the studies

presented herein, the triple and quadruple INSTI resistance mutations emerged in two different patients infected with subtype A and D HIV-1 (respectively) and failing a RAL-based treatment regimen. The high level cross resistance to BIC and CAB by this patient-derived HIV-1 has been confirmed with the same set of mutations in a study screening for BIC and CAB using a subtype B HIV-1 with these mutations introduced *in vitro* (459). Despite high level resistance to both drugs by the RAL selected primary mutations, Q148KR, G140A, E138KA and S147G, the fold-change cross resistance to CAB was more pronounced. BIC, compared to CAB appears to be better accommodated in the binding pocket of IN even with these mutations, possibly due to the oxazepane ring attached to metal chelating scaffold of BIC (651). The superiority of BIC compared to CAB to inhibit viral replication in viruses carrying substitutions at G140 and Q148 has also been reported in studies of mainly subtype B viruses (457,459,647).

Multiple INSTIs-resistance mutations (>3) emerging in HIV-1 infected patient is rare but its detection during RAL failure is not surprising in highly cART experienced patients, especially if RAL failure is prolonged due to limited virological monitoring common in LMICs (646,648). Despite increasing reporting of patients failing INSTIs with multiple primary mutations in non-B HIV-1 subtypes, we found most occurrence of Q148HKR and associated primary mutations to be in subtype B viruses. This may be associated with early use of INSTIs in high income countries where subtype B virus is predominant. The HIV-1 genotypes with Q148HKR in association with multiple INSTIs primary mutations is currently < 3% in Uganda and 5.2% in non-B HIV-1 subtypes which encourages use of BIC and CAB, but increased INSTIs resistance surveillance will be required as INSTIs become accessible in LMICs.

IN-catalyzed integration into cellular DNA is a two-step process of 3′-processing and strand transfer process and is required for viral replication and infectivity (672). IN-resistance mutations including N155H and E138K and added mutations reduce efficiencies of 3′ processing and strand transfer activities which impairs HIV-1 integration capacity (437,440). We find that all IN-recombinant viruses tested exhibited <50% capacity to integrate into cellular DNA which may explain our previous observation of loss of replication fitness associated with these viruses (649).

Conclusions

Emergence of multiple INSTIs-resistance mutations after prolonged virological failure on RAL presents a huge threat to the efficacy of BIC, CAB, and DTG regimens, the latter previously reported. (649) BIC and CAB retain potency in patients carrying most single primary INSTI resistance mutations with or without secondary mutations. However, with common use of RAL-based regimens for third line treatments, failure to a prolonged RAL-based treatment as well as spread of RAL resistance viruses carry multiple INSTI resistance mutations could impact the use of these second generation INSTIs. Given the infrequent use of drug resistance genotyping in almost all LMICs, we have not determined the prevalence of the specific INSTI drug resistance genotypes in both individuals failing INSTI treatment or in treatment naïve population.

Chapter 6

6 Detection of novel HIV-1 drug resistance mutations by support vector analysis of deep sequence data and experimental validation

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6.1 Preface

Based on our previous studies, only about 5% of HIV-1 infected patients in Uganda have drug resistance mutations (DRMs) which could impact efficacy of dolutegravir (DTG) especially when used as salvage therapy. Contrary, over 50% of patients failing raltegravir (RAL)-based regimen have resistant viruses to RAL and elvitegravir (EVG). In addition, around 38% of patients failing RAL-based regimen lack any DRM associated with reduced RAL susceptibility. These patients still fail on treatment which could be related to poor adherence and/or due to a novel resistant mutation(s) currently unrecognized in HIV-1 drug resistance interpretation algorithms.

Support vector machine (SVM) analysis can be used to identify association between groups of population sharing specific traits. SVM was employed to identify novel resistance pathways to RAL resistance in patients found not to harbor any putative DRMs using deep sequencing data. After generation of plasmids with these candidate mutations, recombinant viruses were made and tested for susceptibility on RAL and DTG. In the chapter presented herein, Dr. Mariano Avino the lead author for the manuscript carried out computational analysis for the deep sequencing data. I recruited the patients for the study, carried out all the laboratory assays, and analyzed Sanger sequencing and NGS data for the study.

6.2 Abstract

The global HIV-1 pandemic comprises many genetically divergent subtypes. Most of our understanding of drug resistance in HIV-1 derives from subtype B, which predominates in North America and Western Europe. However, about 90% of the pandemic represents non-subtype B infections. Here, we use deep sequencing to analyze HIV-1 from infected individuals in Uganda who were either treatment-naive or who experienced virologic failure on ART without the expected patterns of drug resistance. Our objective was to detect potentially novel associations between mutations in HIV-1 integrase and treatment outcomes in Uganda, where most infections are subtypes A or D. We retrieved a total of 380 arcHIV-1ed plasma samples from patients at the Joint Clinical Research Centre (Kampala), of which 328 were integrase inhibitor- naive and 52 were RAL-based treatment failures. Next, we developed a bioinformatic pipeline for alignment and variant calling of the deep sequence data obtained from these samples from a MiSeq platform (Illumina). To detect associations between within-patient polymorphisms and treatment outcomes, we used a support vector machine (SVM) for feature selection with multiple imputation to account for partial reads and low-quality base calls. We subsequently introduced the I203M, I208L mutations through site-directed mutagenesis, and used patient-derived integrases carrying I208L or I203M and I208L to evaluate susceptibility of these mutants to INSTIs *in vitro*. Recombinant viruses with mutations, I203M, I208L or I203M and I208L were generated and phenotypically tested to determine susceptibility to RAL and DTG in TZM-bl cells. Novel resistance mutations, I208L, I203M or I203M and I208L identified by SVM did not show reduced susceptibility to RAL or DTG compared to wild-type virus with 1.3-1.8-fold and 1.1-1.4-fold observed, respectively.

Author summary

There are many different types of HIV-1 around the world. Most of the research on how HIV-1 can become resistant to drug treatment has focused on the type (B) that is the most common in high-income countries. However, about 90% of infections around the world are caused by a type other than B. We used next-generation sequencing to analyze samples of HIV-1 from patients in Uganda (mostly infected by types A and D) for whom drug treatment failed to work, and whose infections did not fit the classic pattern of adaptation based on B. Next, we used machine

learning to detect mutations in these virus populations that could explain the treatment outcomes. Finally, we experimentally added two candidate mutations identified by our analysis to a laboratory strain of HIV-1 and confirmed that they conferred drug resistance to the virus. Our study reveals new pathways that other types of HIV-1 may use to evolve resistance to drugs that make up the current recommended treatment for newly diagnosed individuals

6.3 Introduction

There are currently six classes of antiretroviral drugs approved for treatment of HIV-1 infection, with protease inhibitors (PIs) and nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs) in most widespread use (673). Integrase strand transfer inhibitors (INSTIs) are a more recent class of antiretroviral drugs targeting the virus-encoded integrase (IN) protein, which is responsible for inserting complementary DNA derived from the viral RNA genome into the genome of the host cell (674). INSTIs are increasingly being used for individuals in low and middle-income countries (LMICs) for whom first- and second-line antiretroviral treatment (ART) regimens have failed, due to the emergence of drug resistance mutations (DRMs) to the PIs, NRTIs and/or NNRTIs that comprise these regimens (642). With the exception of boosted PIs, there is typically a greater genetic barrier for HIV-1 to develop resistance to second-generation INSTIs, such as dolutegravir (DTG) and bictegravir (BIC), relative to other drugs (671,675). Even so, there are multiple well-characterized mutations conferring major and accessory resistance to INSTIs (676), where we employ the Stanford HIV-1 Drug Resistance (HIV-1db) guidelines for categorizing DRMs (677). Most DRMs with major effects cause some level of cross-resistance to all drugs in this class— DTG, BIC, raltegravir (RAL) and elvitegravir (EVG) — with higher-level resistance to RAL and EVG compared to DTG and BIC.

Until recently, HIV-1 drug resistance studies have generally focused on individuals receiving ART in high income countries. The expansion of ART to over 18 million worldwide has turned attention to finding affordable methods for scaling up treatment monitoring and drug resistance testing. With the high volume of tests, some LMICs have adopted drug resistance genotyping by next generation sequencing (NGS) technologies (678,679), in place of the more common but less scaleable Sanger sequencing approaches (680). In addition to the ability to multiplex large numbers of patient samples into a single run (681), NGS has an added advantage of deep sequencing — where the same region of the virus genome is covered by sequences from hundreds or thousands of individual viruses in the sample — making it possible to reproducibly identify minority HIV-1 variants below the detection threshold of Sanger sequencing (682). Despite their low frequencies within patients, these minority variants have clinical significance as they can

anticipate the emergence of drug resistance and treatment failure (683). Deep sequencing analysis of clinical HIV-1 samples in LMICs also provides a unique opportunity to identify potentially new HIV-1 polymorphisms associated with drug resistance in diverse HIV-1 strains (684–686). These opportunities also present significant bioinformatic challenges. Enormous amounts of sequence data must be processed accurately and efficiently, where sequencing error rates still exceed conventional Sanger methods (687). The task of identifying novel associations between treatment outcomes and minority variants in diverse HIV-1 populations remains an open problem, and research has focused largely on HIV-1 coreceptor tropism (688,689) in populations predominantly affected by subtype B..

The global diversity of HIV-1 is structured into four phylogenetic groups, denoted by letters M-P (690). The vast majority of infections worldwide are caused by group M viruses, which are further separated into subtypes that have distinct geographic distributions, possibly owing to early 'founder effects' in sub-Saharan Africa (691). The majority of research on DRMs has historically been carried out on HIV-1 subtype B, owing in part to the predominance of this subtype in North America and western Europe — this subtype represents only about 10% of the global HIV-1 pandemic (692,693). Fortuitously, clinical outcomes on first- and second-line ART appear to be largely independent of HIV-1 subtype (483,484,694). However, several studies (reviewed in (695) have shown that non-subtype B infections can accumulate DRMs in response to treatment along mutational pathways that are distinct from subtype B. For example, a novel DRM in HIV-1 RT (V106M) has been reported to confer resistance to the NNRTI efavirenz (EFV) that is characteristic of HIV-1 subtype C (513). More recently, investigators determined that the HIV-1 integrase mutation G118R confers a high level of resistance to RAL in the circulating recombinant form CRF02 AG, where the glycine is highly conserved across subtypes (493). According to that study, G118R had only been previously observed in cell culture on exposure to another second-generation INSTI (MK- 2048).

Historically, Uganda has had one of the highest burdens of HIV-1/AIDS in the world, with an estimated 1.3 million people living with HIV-1. The majority of infections in Uganda are caused by HIV-1 subtypes A and D, followed by A/D inter-subtype recombinants and subtype C (696). With increasing access to ART, the transmission of DRMs is becoming increasingly common with

an estimated 5% to 9% of treatment- naive individuals carrying at least one primary DRM (488). The majority of individuals starting ART in Uganda are prescribed a first-line regimen based on EFV, tenofovir (TDF) and a second NRTI, whereas almost no one had received the World Health Organization-recommended (666) INSTI + 2 NRTIs initial regimen that is more common for first-line therapy in higher income settings (540,697). DTG was recently introduced into new first line treatment regimens across sub-Saharan Africa, but treatment with any INSTI still represents less than 1% of active first line treatments in LMICs (698). In most LMICs, INSTIs have generally been reserved for those requiring a third-line treatment regimen, with RAL-based regimens being quite successful in treatment-experienced individuals with multidrug-resistant HIV-1 (628,699,700). Several mutational pathways reducing susceptibility to RAL have been described, including the major DRMs T66K, Y143R, Q148H/K/R, and N155H in HIV-1 integrase (433,617,671,701). Notably, all of these studies were carried out in predominantly (≥90%) HIV-1 subtype B cohorts or *in vitro* with a subtype B laboratory clone.

Here, we describe a bioinformatic approach to detect potential novel DRMs from NGS data sets that include all within-host polymorphisms above a frequency of 1%. We have applied this method to HIV-1 NGS data from a cohort of individuals with HIV-1 non-subtype B infections in Uganda, of whom a subset had experienced treatment failure on RAL-containing salvage regimens. Addition- ally, we have experimentally verified the resistance effects of the novel DRMs predicted by our bioinformatic analysis by drug susceptibility assays *in vitro*, and characterized these mutations in structural models of HIV-1 integrase.

6.4 Methods

Data collection

The study samples were collected from the Center for AIDS Research Laboratory at the Joint Clinical Research Centre (JCRC) in Kampala, Uganda (540). Written informed consent was provided by all study participants. Ethical approval was obtained from JCRC and University Hospitals Cleveland Medical Center/Case Western Reserve University Institutional Review Boards (EM-10- 07 and 10-05-35). All investigations have been conducted according to the principles expressed in the Declaration of Helsinki. Patient samples were assigned to one of four categories based on treatment history and clinical outcome records in the JCRC database: treatment- naive, first-line treatment failures, second-line treatment failures, and treatment failures on RAL-based salvage regimens (RAL failure). Treatment failure was defined by the presence of either a viral load above 1,000 copies/mL and/or a CD4 cell count below 250 cells/mm^3 in the period following treatment initiation. Although current definitions of treatment failure tend to focus on viral load measurements, we retained the criterion based on CD4 cell counts for consistency with historical practice in this treatment population.

RNA extraction and PCR amplification

For each sample, viral RNA was extracted from 200μL of plasma using a QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription of the full-length HIV-1 integrase (IN)-coding region from extracted viral RNA and amplification was performed with the sense primer RTA9F (5-TATGGGGAAAGACTCCTAAATTTA-3) and antisense primer 3Vif (5-AGCTAGTGTCCATTCATTG-3) using a Superscript III single RT-PCR system with Platinum Taq DNA polymerase kit (Thermo Fisher Scientific) as per the manufacturer's instructions. The complementary DNA product was purified using a Quant-iT Picogreen dsDNA assay kit (Thermo Fisher Scientific) and quantified using a Qubit fluorometer (Thermo Fisher Scientific). The region encoding integrase was amplified in two parts by nested PCR using the following sets of primers: (1) sense primer INTF1B (5'-AGGTCTATCTGGCATGGGTACC -3') and antisense primer INTR1B (5'-GATTGTAGGGAATTCCAAATTCCTGCT-3'); (2) sense primer INTF2B2 (5'-CAGGAATTTGGAATTCCCTACAATCCCC-3') and antisense primer INFR2B4

(5’-TGTC TATAAAACCATCCCCTAGCTTTCCC-3’).

Library preparation and deep sequencing

Two overlapping IN-PCR regions corresponding to the 288 amino acids of HIV-1 IN were sequenced with the MiSeq NGS platform (Illumina). The amplicons were purified with Agencourt AMPure XP (Beckman Coulter) and quantified using the Quant-iT Picogreen dsDNA assay kit (Thermo Fisher Scientific), prior to adding adapters using the Nextera XT sample prep kit (Illumina) with dual indexing for a maximum of 384 unique tags. The resulting libraries were quantified, normalized and pooled for paired-end sequencing (2×300 nt) on the Illumina MiSeq platform. Signal processing, base calling and structural variant analysis were performed with the MiSeq Re- porter Software (version 2.6, Illumina). We deposited the unprocessed FASTQ data in the National Center for Biotechnology Information (NCBI) Short Read ArcHIV-1e (BioProject accession number PRJNA554675).

Site directed mutagenesis of I203M and I208L

The I203M and I208L mutations were created in HIV-1 INT gene from pREC-NFL (NL4-3) back- bone using in-house site directed mutagenesis protocol. Briefly, the HIV-1 IN coding regions were amplified with the following primers: Vif 3 reverse 1 (5′-GTCCTGCTTGATATTCACACC-3′); INTREXT (5′-AATCCTCATCCTGTCTAC-3′); and INTFEXT1 (5′-AGAAGTAAACATAGTAACAGACTCACA-3′). The I203M mutation was created using sense primer 5′-gcaggggaaaga atagtagacATGatagcaacagacatacaaac-3′ and the antisense primer 5′-gtttgtatgtctgttgctatCATgtctac tattctttcccctgc-3′); I208L was created using the sense primer 5′-gaatagtagacataatagcaacagacTTG caaactaaagaattacaaaaa-3′ and antisense primer 5′-tttttgtaattctttagtttgCAAgtctgttgctattatgtctactatt c-3′. The presence of the mutation in the plasmid and the propagated virus was confirmed by PCR followed by Sanger sequencing.

Cells and antiviral compounds

TZM-bl, U87.CD4.CXR4 and HEQ293T cell lines were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, U.S.) (702). All cell lines were maintained in Dulbecco modified Eagle medium

(DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin-streptomycin. In addition, U87.CD4.CXR4 cells were maintained in the presence of 300 μg/ml G418 (an amino- glycoside antibiotic) and 1 μg/ml puromycin (Invitrogen, Carlsbad, CA). All cell lines were sub- cultured every 3-4 days at 37˚C under 5% CO_2. The TZM-bl cells contained reporter luciferase and β -galactocidase reporter genes that were activated by expression of HIV-1 *tat*. DTG and RAL were provided by Gilead Sciences (Foster City, CA, USA).

Construction of HIV-1 INT chimeric viruses

HIV-1 full length integrase PCR products were cloned into pREC NFL IN/URA3 vector and *Saccharomyces cerevisiae* MYA-906 cells (ATCC) using the yeast homologous recombination-gap repair system (638). Following homologous recombination, plasmids were extracted from the yeast cells and transformed into electrocompetent *Escherichia coli* Stbl4 cells (Invitrogen). Plasmids were extracted using Qiagen miniprep kits and plasmid DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific). The presence of the mutation in the generated plas- mid was confirmed by sequencing. Chimeric pREC NFL INT plasmids were co-transfected into HEK293T cells (3×10^4 cells/well) along with the complementing plasmid pCMV cplt using Fu- gene 6 reagent (Promega, Madison, WI) as described previously (638). Virus was then propagated on U87.CD4.CXCR4 cells as described (638).

Drug susceptibility assay in TZM-bl cells

HIV-1 susceptibility to DTG and RAL was determined using TZM-bl cells. Briefly, 20,000 cells per well were exposed to wildtype (WT), and mutant HIV-1 in presence of 10-fold dilutions of DTG or RAL (100 μM to 10^{-7} μM) and polybrene (1mg/ml) in 96-well tissue culture plates (Corning). The amount of virus added to each well was normalised to a multiplicity of infection (MOI) of 0.05 based on the infectious titer. After 48 hr incubation at 37°C and 5% CO_2, the infectivity of viruses was quantified using Galacto-Star chemiluminescent reporter gene assay and detection was done using Cytation 5 plate reader (BioTek USA). The fold changes in the effective concentrations for 50% inhibition (EC_{50}) and standard errors of the mean (SEM) were calculated based on two sets of experiments, each performed in quadruplicate. Drug sensitivity curves were generated using nonlinear regression curve fitting features of GraphPad Prism 8.0 software (GraphPad Soft- ware, Inc., San Diego, CA). Drug resistance is presented as fold change

in EC_{50} between WT and mutant viruses.

Sequence analysis

We processed the FASTQ files generated by the Illumina MiSeq platform using a customized version of the MiCall pipeline (https://github.com/PoonLab/MiCall-Lite) (687). First, the pipeline extracts the empirical φ X174 error rates from the 'ErrorMetricsOut' binary InterOp file associated with the MiSeq run, and then censors bases in the FASTQ files associated with problematic cycle-tile combinations with error rates exceeding a cutoff of 7.5%. Next, the program cutadapt (version 1.11) (703) was used to filter the FASTQ read data for Illumina adapter sequences. The pipeline subsequently used the alignment program Bowtie2 (version 2.2.6) (704) to map the paired- end read data to the full-length sequence encoding HIV-1 integrase of the HXB2 reference genome (Genbank accession K03455). This preliminary mapping stage was followed by the iterative re- mapping of reads from the original FASTQ files to new reference sequences, which were progressively updated with the plurality consensus of reads that were successfully mapped in the previous iteration (687). A mapping quality score cutoff of $Q = 20$ was applied at this stage to filter ambiguously mapped reads. The primary outputs of the pipeline included the sample-specific nucleotide consensus sequence, coverage maps, and the matrix of amino acid frequencies in the coordinate system of the HXB2 reference; insertions relative to this reference coordinate system were written to a separate output file.

We used a Python script to filter the amino acid frequency matrices generated by the pipeline described above, using a minimum coverage threshold of at least 1,000 mapped reads per amino acid. First, any matrix corresponding to a pair of FASTQ files was discarded if the overall number of reads mapped to the sample-specific consensus sequence was below this threshold. Next, any individual amino acid position below this coverage threshold was coded as missing data in the remaining frequency matrices. Additionally, any sites with discordant amino acid frequencies within the overlapping region of the two amplicons — *i.e.*, where the frequency of an amino acid exceeded the threshold in one amplicon but not the other — was also coded as missing data. Next, the script converted the amino acid frequency data for each sample into a long binary vector that indicated whether each of the 20 amino acids was observed above a frequency threshold of 1% at every position in the integrase gene; these outputs are herein denoted the low-threshold (LT) data set. This dichotomization step was repeated at a frequency threshold at 20% to produce the high-

threshold (HT) data set. Hence, the amino acid frequency data from the aligned short reads were encoded into two presence-absence matrices, each comprising $20 \times 288 = 5,760$ variables. (columns) and one row for every pair of FASTQ files. Similar dichotomization approaches (*e.g.*,sparse binary encoding (688) have previously been used for feature selection analyses involving amino acid polymorphisms (688,705). All subsequent analyses were replicated across these two data sets.

Data imputation

A substantial number of patient samples (Supplementary Table S1) were sequenced more than once on the MiSeq platform to take advantage of the large number of index combinations and sequencing yield of this instrument. In other words, the number of rows in the presence-absence matrix produced by the previous step was greater than the number of patient samples. To incorporate the entire data collection without unnecessarily and arbitrarily discarding or pooling repeated measurements, we randomly down-sampled redundant rows to obtain a reduced presence-absence matrix with one row per sample and repeated this procedure to yield 10 replicate matrices. All subsequent analyses were replicated across these matrices.

The next stage of our analysis employed a support vector machine (SVM) classifier to identify putative associations between amino acid polymorphisms and RAL failure. Although extensions of SVMs have recently been developed to handle missing data (706), the prevailing approach is to use a generic method to impute missing values prior to the SVM analysis. We used multivariate imputation by chained equations as implemented in the *R* package *mice* (707). Based on the overall proportion of missing observations in our data sets (3.2% for the LT data set and 2.9% for the HT data set), the recommended minimum number of imputations was 3 (708). We decided to generate 5 imputed data sets for each of the 10 normalised data sets from the previous section. Further, we duplicated this approach for the LT and HT data sets for a total of $5 \times 10 \times 2 = 100$ imputed data sets. To speed up the multivariate imputation, we used the *quickpred* variable selection procedure implemented in the *mice* package to filter the data for potentially significant predictors based on a simple correlation statistic. We excluded sites with an absolute correlation with the group labels below 50% and output the remaining variables to a preliminary predictor matrix. Each imputation was run for 20 iterations instead of the default 5 iterations, and convergence was visually assessed using the trace line plots of estimates against iteration numbers. To increase the robustness of

results from the SVM analysis, we filtered amino acid features with non-zero weights (based on their incorporation into support vectors) by a minimum frequency of 80% across imputations, *i.e.*, at least 40 out of 50.

SVM analysis

The preceding imputation step yielded 100 large presence-absence matrices encoding the observed HIV-1 integrase amino acid polymorphisms across baseline and treatment failure samples. We analyzed each imputed data matrix with a support vector machine (SVM) in which the samples which was encoded by +1 and −1, respectively, as stipulated by the soft-margin linear SVM model were mapped to a high-dimensional feature space based on the presence or absence of amino acids, (709)[52]. Our samples were recategorized into two groups of outcomes (labels): samples from patients who experienced treatment failure on a RAL-based regimen (abbreviated to 'RAL failure'); and samples from treatment-naive patients and patients who experienced treatment failure on first- and second-line regimens without integrase inhibitors ('RAL naive'). An SVM attempts to locate the hyperplane, defined by a subset of data points (the support vectors), that most effectively separates the training data into the two groups. We performed an SVM analysis with the *svm.fs* function from the *R* package *penalizedSVM* (710) using an L1-norm penalty. Compared to an unpenalized SVM, this penalty function aggressively zeroes-out the coefficients associated with features that are less informative for classifying the data, and thereby provides a framework for feature selection (711). To calibrate the λ tuning parameter of the SVM model, which controls the severity of penalizing data points that cross the margin of the hyperplane, we used a discrete grid search to determine the optimal λ with minimal misclassification error by 5-fold cross-validation (712). After training and cross-validation, we generated the final SVM model for the entire data set using the optimized λ parameter. For each of the 100 data sets, we extracted the average feature weights and counts from the SVM results.

To corroborate the assignments of the most positive or negative feature weights to specific amino acids per treatment group, we calculated the odds ratios to quantify the statistical associations between the amino acid and outcomes (group labels). We calculated odds ratios and 95% confidence intervals by unconditional maximum likelihood estimation (Wald method) as implemented in the *R* package *epitools*, adding a fixed $n = 0.5$ value to every single cell of its contingency table to avoid a division by zero error (Haldane-Anscombe correction (713).

Subsequently, we averaged the results for each feature across all 100 data sets. Additional details on the sequence processing and SVM analysis are provided as Supplementary Text S1.

Drug resistance prediction

To obtain resistance predictions from the sequence data from the IN coding region, we generated a consensus sequence at a polymorphism threshold of 20%, such that any position with two or more nucleotide frequencies above this threshold was encoded as an ambiguous base using the corresponding IUPAC symbol. Further, we censored all positions with fewer than 50 mapped reads as missing data – this threshold was less stringent than the minimum number of mapped reads (1000) required for positions to be carried over to the SVM analysis, because the objective of the latter was to detect associations with polymorphisms at a minimum frequency of 1%. Since a minimum of 10 mapped reads (1% × 1000) was required to be interpreted as a real polymorphism, the same number of mapped reads was required to influence the consensus sequence at a cutoff of 20% (50 reads × 20% = 10 reads). Drug resistance prediction scores on the resulting consensus sequences were obtained for RAL using the Stanford HIV-1db algorithm version 8.4 (updated 2017- 06-16) (677).

Subtype and phylogenetic analysis

We used the same consensus sequences generated for drug resistance prediction to predict subtypes and reconstruct the phylogeny. We used the SCUEAL algorithm in HyPhy (608) to generate subtype classifications and detect inter- and intra-subtype recombination. Next, we excluded predicted recombinant sequences and sequences that were classified as circulating recombinant forms (CRFs), and generated a multiple sequence alignment from the remaining sequences using MUSCLE (version 3.8.425) (714). This alignment also incorporated the HIV-1 reference sequences curated by the Los Alamos National Laboratory (LANL) HIV-1 Sequence Database (http://www.HIV-1.lanl.gov) for subtypes A1, C, D and G, where this selection of references was based on subtyping results from this study population. The alignment was manually inspected and refined in AliView (version 1.19-beta-3) (610). We used jModelTest (version 2.1.10) (612) to select the most effective nucleotide substitution model based on the Akaike Information Criterion (AIC). Finally, we used PhyML (version 20160207) (715) to reconstruct a phylogenetic tree by maximum likelihood un- der the AIC-selected model with the default bootstrap support

analysis (1,000 replicates). The tree was visualized and manually annotated for subtypes in FigTree (version 1.4.2, A. Rambaut, http://tree.bio.ed.ac.uk/software/figtree).

Structural analysis

The α-helix that connects the catalytic core domain (CCD) to the C-terminal domain (CTD) of the HIV-1 integrase protein is not resolved in the available DNA bound three-dimensional (3D) structure (PDB ID 5U1C). We therefore modelled the coordinates of the missing α-helix region (a total of 22 amino acids derived from PDB ID 1EX4) into one monomer (chain A) of the original structure (PDB ID 5U1C) using the program MODELLER (version 9.21) (716) to create an extended structure (PDB ID 5U1C extended). Using the extended structure as template, we built structural models of the I203M, I208L and combined I203M and I208L mutations respectively. The protein refinement program 3Drefine was used for energy minimization and optimization of all models (717). Furthermore, we used the molecular structure visualization program PyMOL (version 1.7.2.1, http://pymol.org; Schrödinger, LLC) to visualize the sites where the I203M and I208L mutations are located on the 3D structure (PDB IDs 5U1C, 5U1C extended and 2B4J) of the HIV-1 integrase protein (91).

6.5 Results

Data collection

We obtained plasma samples for a total of 380 patients receiving treatment for HIV-1 infection at the Joint Clinical Research Center in Kampala, Uganda, for genotypic drug resistance testing by deep sequencing on the Illumina MiSeq platform. Of the 328 samples from INSTI-naive individuals (RAL naive), 85 samples were categorized as treatment-naive, 127 as first-line treatment failures, and 116 as second-line treatment failures. The remaining 52 samples (14% of total) were obtained from individuals who had experienced treatment failures on raltegravir-based salvage regimens (RAL failure). From these samples, we generated a total of $n = 524$ paired FASTQ files with a mean of 83,185 reads per pair. Using the MiCall pipeline (687), which iteratively re-maps read data to update sample-specific reference sequences, we mapped an average of 81,189 reads (97.6% of the raw totals) to the HIV-1 IN coding region from each sample. This pipeline enforced a number of coverage and quality filtering criteria (see Methods), including a minimum requirement of 1,000 read coverage per amino acid position. Consequently, we discarded $n = 7$ FASTQ files due to insufficient numbers of reads that mapped to the HIV-1 reference.

The φ X174 control error rates associated with the two MiSeq runs used for these sequencing experiments displayed the typical exponential decay with increasing cycle number, starting at a median of 0.44% (interquartile range, IQR: 0.32%, 0.96%) and ending at 9.2% (6.7%, 15.6%; Supplementary Figure S1). The overall median error rate was 1.5% (0.49%, 3.7%), which was consistent with previously reported error rates for this platform. Out of a total of 22,800 tile-cycle combinations, 2,095 and 3,360 combinations with an error rate exceeding 7.5% from the respective runs were excluded from further analysis. These combinations were concentrated in the last 100 cycles of the second reads (80.3% and 74.6%). The median sequence length after mapping with soft clips was 498 (IQR 325, 525) nt, indicating that the majority of the dropped base calls due to bad tile-cycles could be compensated by high quality base calls in the first reads at the paired-end read merger step of the pipeline.

A key challenge in detecting genetic associations in deep sequencing data from rapidly-evolving pathogens like HIV-1 is that polymorphisms can be observed at many, if not most, sites. Hypothetically, there exists a frequency threshold that optimally separates polymorphisms caused

by sequencing errors from actual variants with potential clinical significance. Because it was not feasible to replicate all downstream analyses for an exhaustive sample of frequency thresholds, we proceeded with 1% (low threshold, LT) and 20% (high threshold, HT) to dichotomize the amino acid frequency data into binary presence/absence values. These values were chosen on the basis of prior information on the expected error rate for this sequencing platform (687) and the detection limit of Sanger sequencing (680,682), respectively.

After excluding reads with low map quality scores and censoring low quality or discordant base calls, we obtained a mean coverage of 18,907 and 19,076 reads per amino acid site for LT and HT data sets, respectively. We restricted our subsequent analyses to amino acid polymorphisms, excluding variation due to insertions, deletions, and premature stop codons. On average, we observed 10^{-4} insertions and 8×10^{-5} deletions per nucleotide, which was within the expected range of indel error rates for this sequencing platform (718). Ambiguous amino acid polymorphisms due to low base quality or incomplete coverage affected a small fraction of the data sets (3.2% and 2.9% for the LT and HT data, respectively). These ambiguities were encoded as missing data and handled through multiple imputation.

Sequence subtyping

Subtyping analysis of the majority consensus sequences derived from the NGS samples confirmed that the majority of samples were assigned to HIV-1 subtypes A ($n = 159$, 49.7%) and D ($n = 70$, 21.3%) as expected for this study population in Uganda. An additional $n = 28$ (8.4%) samples were predicted to be A/D recombinants, and $n = 16$ (4.2%) samples were predicted to be subtype C. The remaining samples were assigned to other inter-subtype recombinants or could not be confidently assigned to a known subtype or circulating recombinant form. We found no significant association between predicted HIV-1 subtypes and RAL-based treatment failure (Fisher's exact test, $P = 0.5$). Variation among sequences was best explained by the general time reversible model of nucleotide substitution with invariant sites and a gamma distribution to model rate variation across sites (GTR+I+G). We reconstructed a maximum likelihood phylogeny under this model to verify the subtype predictions from SCUEAL relative to curated HIV-1 subtype

reference sequences, where sequences assigned to subtypes A, C and D comprised monophyletic clades with high bootstrap support values (>85%).

Drug susceptibility by genotyping

Applying the Stanford HIV-1db algorithm to the NGS consensus sequences to predict resistance to RAL, we confirmed that less than one-third of RAL-based treatment failures (shortened to 'RAL failures') manifested the classical RAL resistance pathways (540). The complete breakdowns of resistance prediction scores by HIV-1 subtype in the RAL naive and RAL failure groups are summarized in Fig. 34. Out of 52 consensus sequences from RAL failure samples, only 14 samples (26.9%) were predicted to have high-level resistance to RAL (score ≥ 60). One of these samples (score 120) harbored the major RAL resistance mutation G140A (in 99.12% of reads) in combination with the accessory mutation E138K (99.1%).

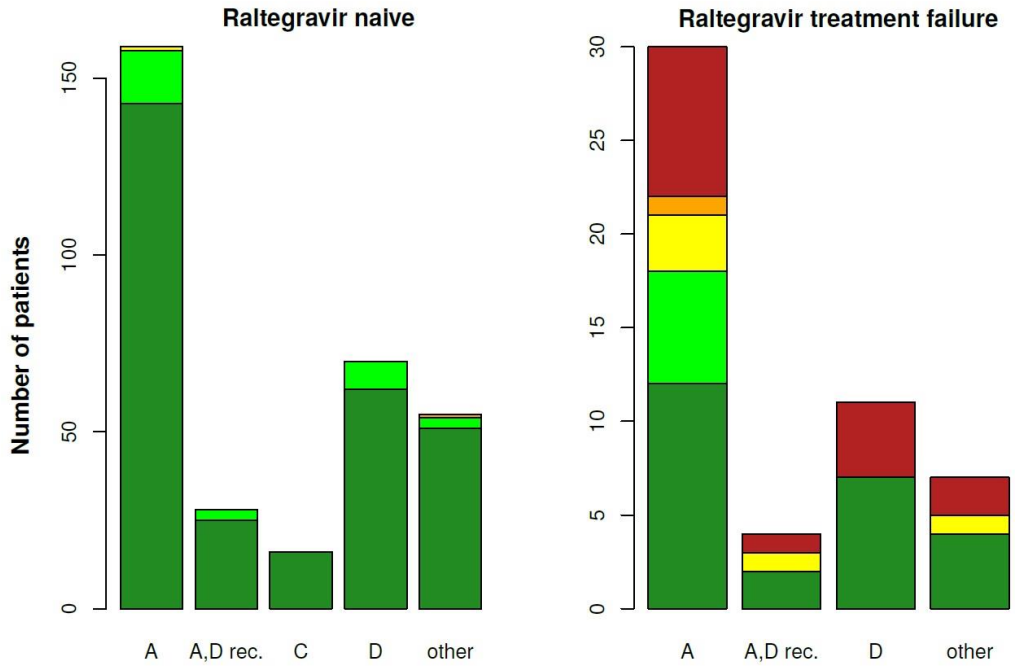

Figure 34. Distribution of RAL resistance predictions on sample consensus sequences by HIV-1 subtype and treatment outcomes. Resistance prediction scores were obtained by the Stanford HIV-1db algorithm [7]. The study population was split into RAL naive (left, n=328) and RAL failure (right, n = 52) patients; accordingly, these plots are on different scales. Each stacked barplot stratifies patients by predicted HIV-1 subtype and categorizes the prediction scores into high-level (60+, red), intermediate (30-59, orange), low-level (15-29, yellow) and potential low-level resistance (10-14, light green), and RAL susceptible (below 10, dark green).

An additional 10 samples carried the major mutation N155H at frequencies between 94.2% and 99.7%, and two samples carried the major RAL resistance mutation T66K (at 93.8% and 62.7%, respectively). Finally, two samples with high RAL resistance scores in this group carried the major mutation Q148R in combination with accessory mutation G163R. This relative lack of expected mutational pathways in the RAL failure group, quantified by the low number of patient samples with resistance prediction scores in the susceptible to low-level resistance range (Fig. 22), motivated a more comprehensive analysis of HIV-1 integrase polymorphisms in the deep sequencing data.

Support vector machine analyses

A key challenge in detecting genetic associations in deep sequence data from rapidly-evolving infections like HIV-1 is that potentially any amino acid may appear at any position. We followed the sparse binary encoding approach in (688) so that every sample was represented by a total of 5,760 binary variables for 20 amino acids at 288 positions in the HIV-1 integrase reference. This is an unwieldy number of predictor variables for conventional association tests like logistic regression. Support vector machines (SVMs (719) were developed to handle this sort of scenario, where the number of observed cases is vastly exceeded by the number of predictor variables, and have been employed in a number of studies of HIV-1 variation (720,721).

We used penalized SVMs to select features (polymorphisms) that most effectively separated patient samples into RAL naive and RAL failure categories. Our results are summarized by the mean feature weights (the relative contributions of different polymorphisms to the separation of labels) for the low (1%) and high (20%) amino acid frequency threshold (LT and HT) data sets, respectively. Figs. 35A (for LT) and 35B (for HT) display these results in comparison to the mean univariate odds ratios (ORs) for polymorphisms selected by the SVM analyses to provide more intuitive measures of association. To account for variation induced by missing data, we excluded features that were selected in fewer than 40 out of 50 imputed matrices for both LT and HT data sets, which disproportionately affected features with average weights close to zero.

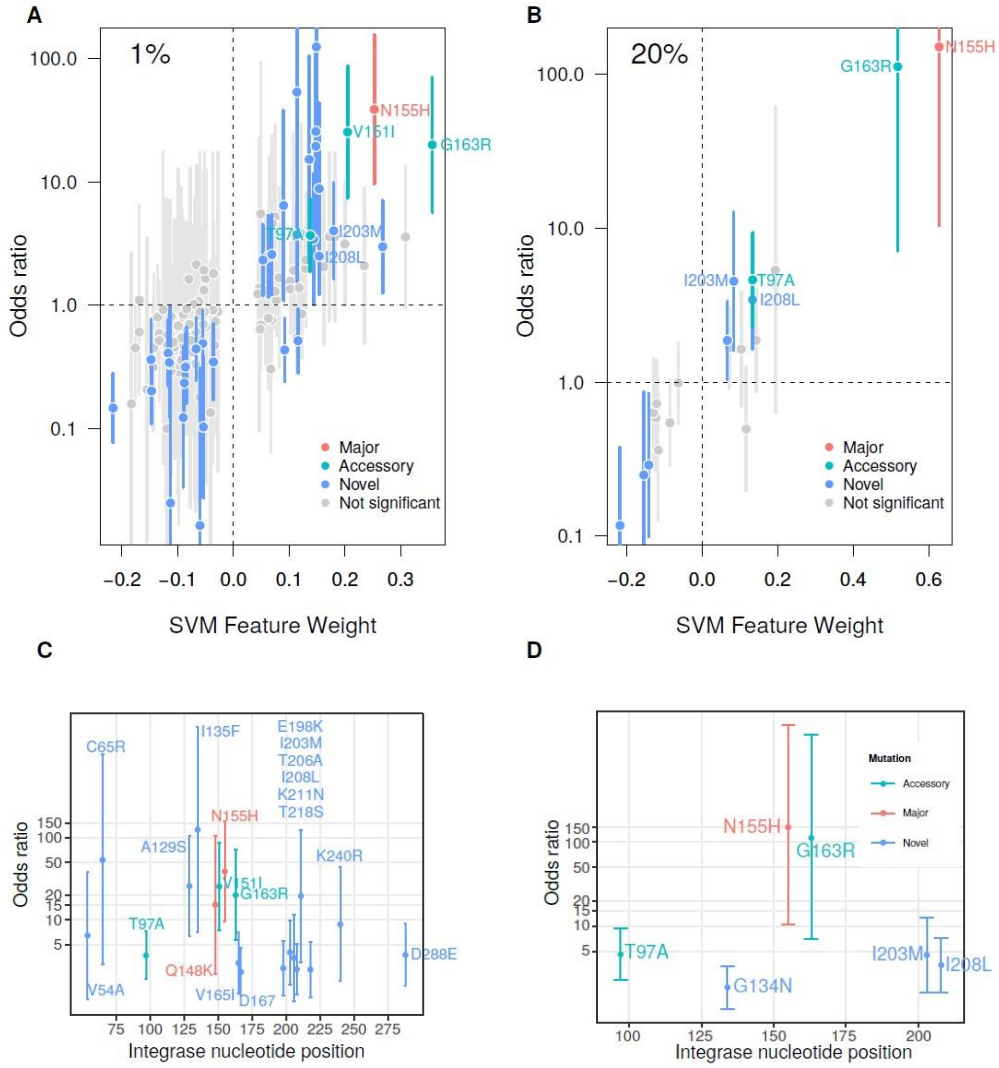

Figure 35. Summary of results from support vector machine analyses. (top) Feature weights and odds ratios for selected polymorphisms in the LT (A) and HT (B) databases. Each point corresponds to an amino acid polymorphism (feature) selected by support vectors. To reduce clutter and identify features robust to missing data, we removed all features selected in fewer than 40 out of 50 imputations. The x-axis corresponds to feature weights, and the y-axis represents the log-transformed mean odds ratio (OR) for each feature against the labels. Vertical lines indicate the empirical 95% confidence interval in ORs. Points are coloured grey if this interval spans 1 (not significant) and otherwise according to mutation categorization, if any, by the Stanford HIV-1 Drug Resistance Database (see inset legend). (bottom) Positive weight selected from the SVM analysis and greater than one Odd ratio polymorphisms for the LT (C) and HT (D) data sets. The HIV-1 integrase reference coordinates are marked along the x-axis, and log-transformed OR mean and C.I. along the y-axis.

In general, we observed strong positive correlations between the SVM feature weights and mean ORs for the LT (Spearman's $\rho = 0.59$, $P < 2.2 \times 10^{-16}$) and HT ($\rho = 0.68$, $P < 2.2 \times 10^{-16}$) data sets.

To identify the most promising features from these results, we filtered the features that were selected in at least 40 imputations and where the mean lower 95% confidence limit in odds ratios was greater than 1. In total, we recorded 663 features selected by support vectors, of which 83 (12.5%) were reproducibly selected in at least 40 imputed matrices. Although the known major RAL resistance mutations T66K (722) and Q148R (723) had positive mean feature weights, they appeared in only 13 out of 50 imputed data sets. Only the major mutation N155H (724) was selected in a majority of imputations (all 50) with the fourth highest mean weight. An additional 8 features were known accessory or minor RAL resistance mutations (T66A (725), L74M (726), T97A (671), V151I (616), N155D (727), E157Q (728), G163R (616) and R263K (729); with the exception of R263K, all were assigned positive weights (Supplementary Table S2). Only G163R, V151I and T97A were selected in a majority (>80%) of analyses, and in fact were selected for all 50 imputed matrices. Overall, our filtering criteria selected the following polymorphisms in descending order of weight: G163R, V165I, N155H, V151I, I203M, T97A, K211N, A129S, D288E, K240R, Q148K, I135F, C65R, I208L and T218S.

The relative locations of these polymorphisms are summarized in Fig. 35C. For instance, we observed a cluster of 6 selected polymorphisms within the interval IN 198 to 218, which is distal to both the integrase active site and RAL binding site.

The higher frequency threshold (<20%) for the HT data set substantially reduced the effective number of amino acid polymorphisms; as a result, there were few features selected in the support vectors (n = 299). Applying the same criteria as above to identify the most significant features yielded the following six substitutions (in decreasing order of weight): N155H, G163R, T97A, I208L, I203M and G134N (Fig. 35D). Of these features, only the primary mutation N155H and secondary mutations T97A and G163R have been previously described.

I203M and I208L alone or together do not confer resistance to INSTIs *in vitro*

Based on these SVM analyses, we selected the novel substitutions I203M and I208L for further

investigation, as they appeared in both lists of the most significant features from the LT and HT data sets. Figure 36 contrasts the intra-host frequencies of I203M and I208L in the context of known major RAL mutations, as defined by the Stanford HIV-1 Drug Resistance Database. These frequency distributions revealed four distinct clusters of mutations among *n* = 30 (58%) patients failing a RAL-based treatment regimen and carrying at least one of these polymorphisms.

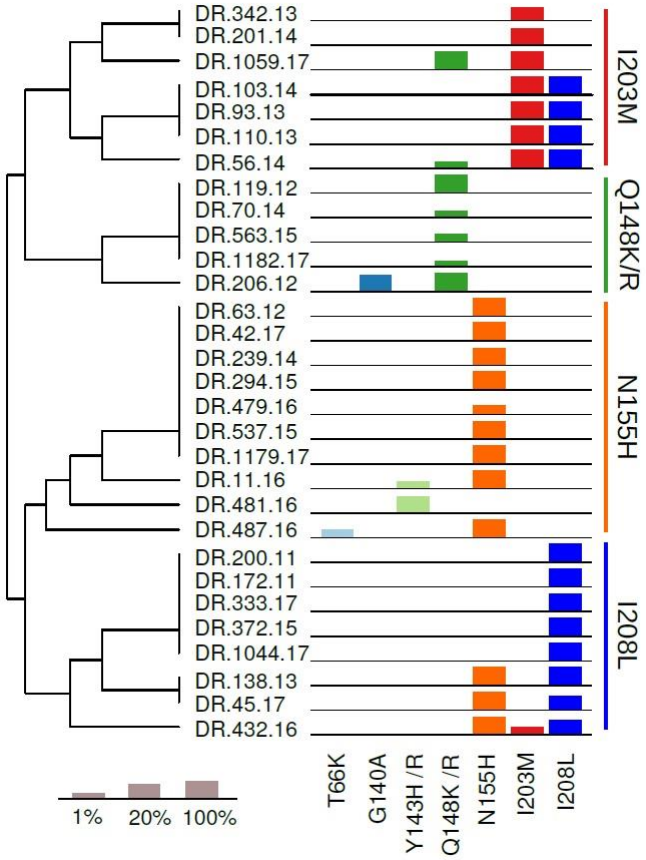

Figure 36. Summary of intra-host frequencies for known major RAL mutations and two candidate mutations I203M and I208L. Samples from n = 30 RAL failures patients carrying at least one of these mutations are each represented by a set of barplots representing mutation frequencies on the right. The height of each bar is proportional to 1+log10(p) for p ranging from 1% to 100% (see legend in bottom-left). The vertical ordering of samples was determined by a hierarchical clustering analysis, with the corresponding dendrogram displayed on the left. Vertical bars have been added on the left to highlight the subsequent specific pathways.

Two clusters of patients in this RAL failure group carried one of two known major RAL resistance mutations Q148K/R or N155H. There were no patient samples containing the polymorphism Q148H. A third cluster comprised samples carrying the mutation I208L, and a fourth carried

I203M and/or I208L to the exclusion of all known major RAL resistance mutations with the exception of Q148K/R. This pattern suggests that I203M and I208L may comprise novel mutational pathways for RAL resistance.

To experimentally validate the predicted effects on resistance to RAL and other INSTIs, the I203M and I208L mutations were placed in the IN of the subtype B laboratory strain NL4-3 (NL4-3_I203M and NL4-3_I208L). Wild type NL4-3, NL4-3_I203M and NL4-3_I208L were propagated and then used in INSTI susceptibility assays as described in the Materials and Methods. I203M or I208L did not impact susceptibility of NL4-3 to RAL or DTG as shown in Figure 37A and B. It is important to note that these two mutations as associated with RAL failure was defined by SVM analyses on Ugandan cohort infected with subtype A and D. As such, we cloned into NL4-3 the HIV-1 subtype A IN from patient DR.110.13 with both I208L and I203M (DR.110.13_I203M+I208L) and from patient DR.372.15 with only I208L (DR.372.15_I208L). These viruses were compared for INSTI susceptibility in TZMbl cells as described. Even in a subtype A IN coding region, the I203M+I208L or I208L did not alter RAL or DTG susceptibility compared to the wild type NL4-3 (Fig.37A and B).

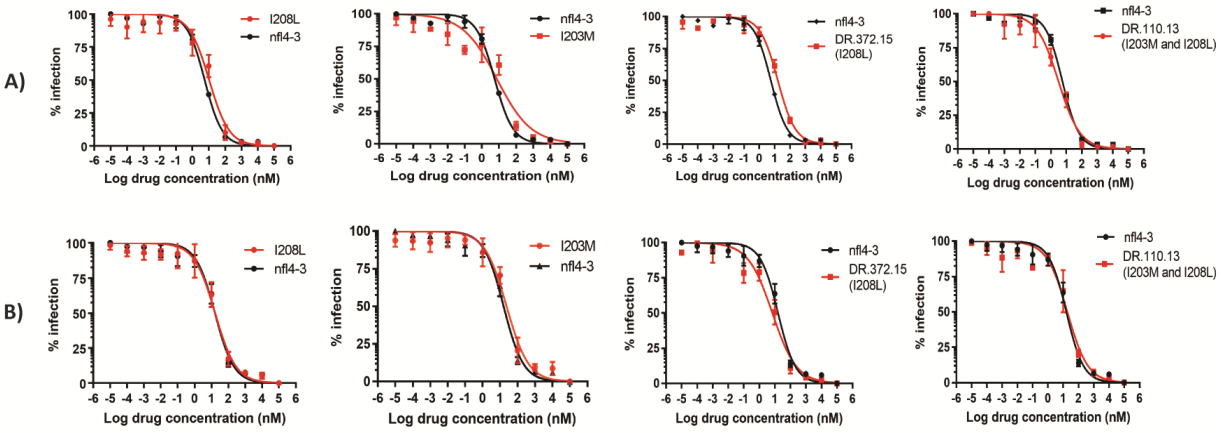

Figure 37. Drug susceptibility assays on the effects of I203M and I208L on INSTI resistance. The top row of plots correspond to raltegravir drug susceptibility curves for NL4-3 (wildtype, in black) and mutants I203M, I208L, or I203M and I208L in either subtype B or A backbone. Similarly, the second row of plots correspond to the dolutegravir drug susceptibility curves for NL4-3 (wildtype, in black) and mutants I203M, I208L, or I203M and I208L in either subtype B

or A backbone. Each point for curves in A-D corresponds to the mean and error bars represent the standard error of at least four replicate assays

I203M and I208L are natural polymorphisms

To assess whether I203M and I208L mutations are present as natural polymorphisms in the general population, we queried the Stanford HIV-1 Drug Resistance Database (HIV-1db; last accessed February 21, 2019) for all available HIV-1 IN sequences (n = 17, 605) from INSTI naive and experienced patients and evaluated the frequencies of these variants in subtypes A, B and D (Table 12). This database comprised n = 1,248 records from RAL-experienced individuals, of which only n =129 were infected with a non-B subtype — in comparison, our study would contribute an additional n = 52 sequences, about 40% of the current number. The frequencies of I203M and I208L in the RAL-naive category in the HIV-1db database were 5.9% and 5.0%, respectively, suggesting these two mutations are natural polymorphisms that circulate at low frequencies (> 0.5%).

Table 12. Frequencies of I203M, I208L and RAL INSTI major mutations in HIV-1 IN stratified by treatment according to Stanford HIV-1 Drug Resistance Database whereas reported RAL-experienced individuals breakdown is reported

	Treatment				
Mutations	naïve	experienced		RAL-experienced	
Total	16029	1575		1248	
I203M	958 (5.9%)	121 (7.7%)	*	96 (6.1%)	*
I208L	779 (5.0%)	128 (8.1%)	***	100 (6.4%)	***
T66K	0 (0%)	4 (0.25%)	***	2 (0.13%)	*
G140A/C/S	3 (0.019%)	350 (22.2%)	***	328 (20.8%)	***
Y143C/H/R	6 (0.037%)	177 (11.2%)	***	170 (10.8%)	***
Q148H/K/R	11 (0.069%)	405 (25.7%)	**	365 (23.2%)	***
N155H	3 (0.019%)	394 (25.0%)	***	359 (22.8%)	***
G140A/C/S + Q148H/K/R	1 (0.006%)	349 (22.2%)	***	325 (20.6%)	***

T66K coverage was 16,027 for naive, I203M and I208L coverage was 15,997 for naive and 1573 for experienced patients. Other major combinations include: G140A/C/S + Y143C/H/R (9 INI), G140A/C/S + N155H (19 INI), G140A/C/S + Y143C/H/R + Q148H/K/R (9 INI), G140A/C/S + Q148H/K/R + N155H (21 INI), G140A/C/S + Y143C/H/R + Q148H/K/R + N155H (2 INI), Y143C/H/R + Q148H/K/R (10 INI), Y143C/H/R + N155H (26 INI), Y143C/H/R + Q148H/K/R + N155H (2 INI), Q148H/K/R + N155H (27 INI), T66K + G140A/C/S + Q148H/K/R (1 INI), INI = INSTI-experienced, RAL = Raltegravir. Fisher's exact tests (experienced vs. naive, RAL- vs. non-RAL-experienced): * = P < 0:05; ** = P < 0:01; *** = P < 0:001.

We noticed only slight but statistically significant increases in these frequencies in association with the RAL-experienced category in HIV-1db (Table 12).

Table 13. Frequencies of I203M and I208L polymorphisms in HIV-1 IN stratified by treatment for this study cohort and by minor allele frequency.

	Low threshold			High threshold		
Mutations	naïve	experienced		naïve	experienced	
Total	328	52		328	52	
I203M	14 (4.3%)	8 (15.4%)	**	8 (2.4%)	6 (11.5%)	**
I208L	39 (11.9%)	12 (23.1%)		28 (8.5%)	12 (23.1%)	*

Low threshold = 1% (LT) and high threshold = 20% (HT). Fisher's exact tests (experienced vs. naive):
* = P < 0:05; ** = P < 0:01; *** = P < 0:001

Although we observed more substantial increases in association with the RAL failure category in our study population (Table 13), this is not directly comparable to the RAL experienced category in the HIV-1db database that comprises an unknown proportion of treatment failures. In addition, we also collected the frequencies of the known major RAL resistance mutations in HIV-1db. These mutations were almost always observed with INSTI-experienced patients, being almost absent in INSTI naive patients (Table 12).

Structural analysis

Based on their location in the primary sequence, I203M and I208L maps near the C-terminal base of the α-helix connecting the C-terminal domain (CTD) to the catalytic core domain (CCD) of HIV-1 integrase in the unliganded structure (91). Figure 38A displays the structure of HIV-1 integrase complexed with viral and host DNA (PDB ID 5U1C) and in which only the I203M position could be mapped. However, as discussed in the methods section, a model of HIV-1 integrase was constructed to create an extension of this structure (5U1C extended), such that when superimposed on the original structure (PDB ID 5U1C), the I208L position was roughly mapped.

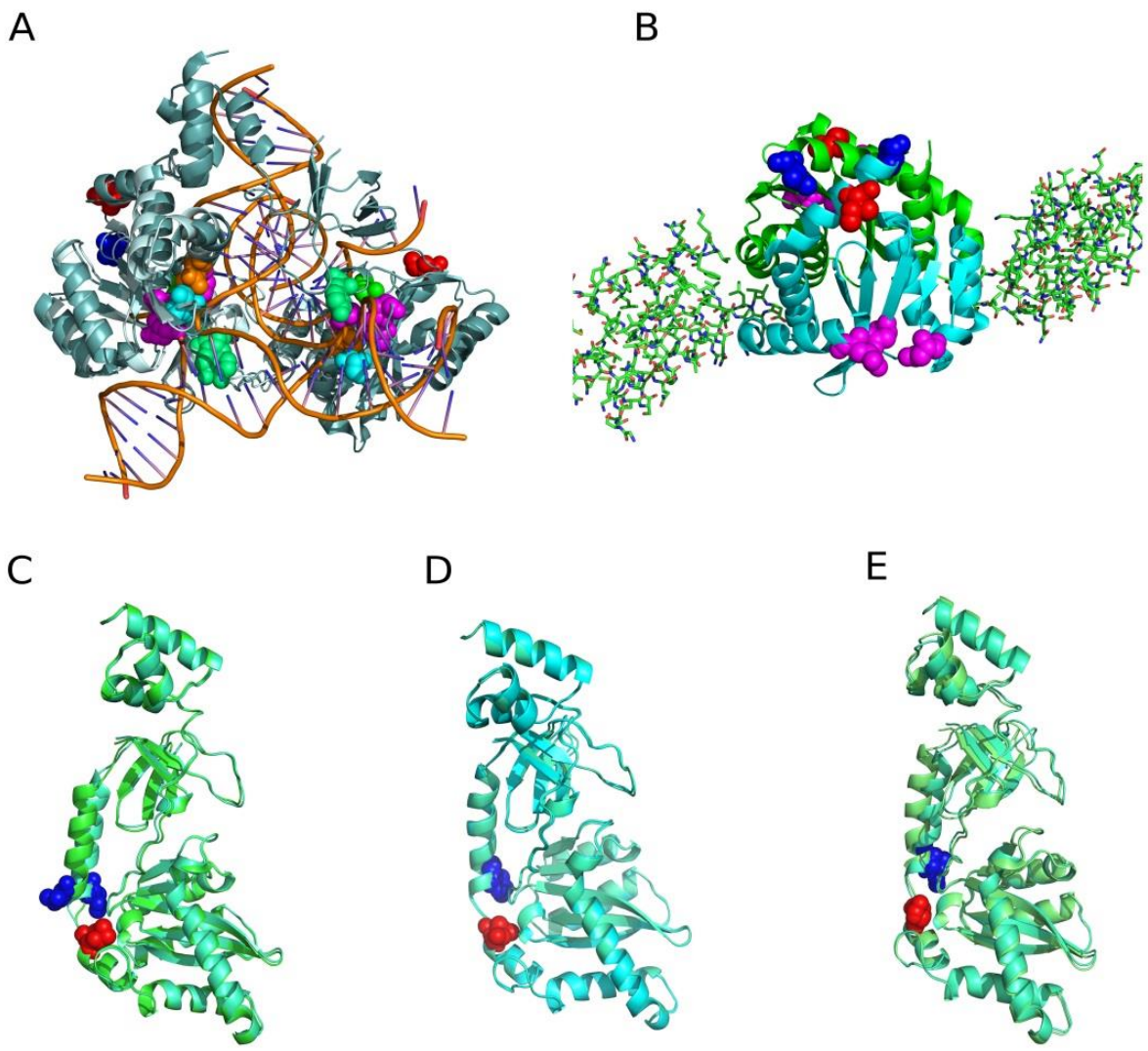

Figure 38. Structural mapping of the novel amino acid replacements. (A) a cryo-electron microscope structure (PDB ID 5U1C) of integrase protein (lightteal) in complex with host and viral DNA. The modelled extended structure (palecyan) is superimposed on chain A to show the missing a-helix where the I208L mutation is located (B) a crystal structure of integrase protein CCD (shown as a green cartoon) bound to the human LEDGEF protein (shown as sticks) (C) the modelled I203M mutant structure (limegreen) of HIV-1 integrase superimposed on the modelled extended wild type structure (greencyan) (D) the modelled I208L mutant structure (aquamarine) of HIV-1 integrase superimposed on the modelled extended wild type structure (greencyan) (E) the modelled I203M and I208M mutant structure (lime) of HIV-1 integrase superimposed on the modelled extended wild type structure (greencyan). All catalytic active site residues are colored purple while the novel mutation sites are colored blue (I203M) and red (I208L) respectively. The known major RAL major resistance mutation sites were colored as follows: T66 (cyan), Y143 (lightgreen), Q148 (green), 155 (orange). These images were generated with PyMOL [81].

From both structures, the I203M and I208L residues are distally located from the catalytic active site of HIV-1 DNA processing, the binding site for most INSTI, as well as the location of most drug resistance mutations (all the known major RAL mutations are also shown in the structure in Fig. 38A). Structural mapping shows that the I203M and I208L mutations were on the opposite side of the C-terminal domain engaged in LEDGEF binding (reference) (Fig. 38B).

Structural modeling of the I203M mutation in HIV-1 integrase shows a conformational change in the α-helix connecting the CCD to the CTD (Figure 38C). Similarly, the structural model having both the I203M and I208L mutations also show a conformational change the α-helix connecting the CCD to the CTD (Fig. 36E). On the other hand, structural modeling of the I208L mutation in HIV-1 integrase shows very little to no conformational changes (Fig. 38D). These residues are closer to the C-terminal domain of HIV-1 integrase that may help coordinate binding to both host and viral DNA (95,730,731).

6.6 Discussion

The majority of our knowledge on drug resistance mutations in HIV-1 comes from studies of subtype B, even though this subtype represents only a small proportion of infections worldwide. In a previous study (540), we observed an absence of known DRMs associated with RAL resistance in half of the HIV-1-infected Ugandans failing a third line RAL-based treatment. This absence could be attributed to either complete non-adherence, or a failure to detect resistance-associated polymorphisms below the threshold of detection of Sanger sequencing (about 20% (682). Based on an initial viral load decrease and adherence tracking during this third line regimen, complete non-adherence resulting in a return of wild type HIV-1 was unlikely for all $n = 51$ patients (540).

Though we cannot retrospectively quantify the extent and impact of drug non-adherence, we can explore the possibility of unique, uncharacterized mutations associated with INSTI resistance with NGS-based genotyping.

Based on previous assay cutoff analyses, the Illumina MiSeq platform is capable of reproducibly detecting mutations at a lower frequency threshold of about 1% (684–686), which confers a substantially improved sensitivity over conventional Sanger sequencing. Setting a frequency threshold of 1% is only meaningful if a sufficient number of virus genomes from the plasma sample are represented in the sequencing library. For instance, if the input number of templates is fewer than 100, then any variant detected at a frequency of 1% or less is likely the result of sequencing error. Our study population comprised patients either sampled prior to initiating ART or following treatment failure. In previous work, we have reported that plasma viral loads averaged about 5.4 $\log_{10}$ copies/mL at baseline (732). Although drug resistance testing in Uganda is requested for patients failing treatment above 1,000 copies/mL, the majority of requests were obtained when viral loads exceed 10,000 copies/mL (averaging 4.8 $\log_{10}$ copies/mL for first-line treatment failure, and >5.0 $\log_{10}$ copies/mL for RAL-based/third-line treatment failure) (540,696).

Our most conservative estimates are that about one-sixth of the viral RNA from 200μL of plasma was transferred from the sample extraction to the RT-PCR reaction mixture, and that about one-half was converted to cDNA. Given that half of the reaction mixture was used for PCR amplification and sequencing, we would expect at least 850 templates on average to be available for NGS. To

evaluate the effect of template resampling on our ability to measure variant frequencies, we can simulate the sampling process assuming that extraction and aliquoting is sampling uniformly at random without replacement, and that sequencing post-amplification is sampling uniformly at random with replacement. For instance, we predict that a variant found in 500 copies/mL (0.5% of the plasma sample) has a 0.03% probability of being sampled to a frequency of 1% or greater under our experimental conditions (2.5% and 97.5% quantiles = 0.26%, 0.77%; $N = 10^6$ replicates). Conversely, a variant in 1500 copies/mL (1.5%) would be sampled at 1% or less with probability 0.93%. We further note that our multiple imputation across repeated NGS of the same samples would have averaged out some sampling variation.

Our NGS analysis of RAL naive and treatment failure samples confirms results from our previous study (540), including the absence of previously identified DRMs in about half of RAL failure cases. The SVM analysis of these data also identified a number of potentially novel mutations associated with reduced sensitivity to RAL. Because this classifier evaluates features by selecting data points (the support vectors) to anchor the hyperplane separating labels, some of these features may be associated by chance with RAL failures due to their linkage to features with direct effects. Consequently, we carried out a *post hoc* odds ratio analysis to evaluate the significance of univariate associations between each feature selected by the SVM and the labels (virologic control versus RAL failure). The combined analyses recovered several known major and accessory mutations conferring resistance to RAL. For instance, we found a highly significant association between the major mutation N155H and patients failing RAL treatment (724), and this mutation was significantly linked with accessory mutations such as V151I (616). A limitation of our SVM analysis was that we selected two frequency thresholds to dichotomize amino acid polymorphisms into binary variables. Although it is possible to directly apply the SVM classifier to continuous variables, dichotomizing the observed frequencies into presence/absence states was necessary to make the analysis computationally feasible. We selected the two frequency thresholds (1% and 20%) to span the range bounded by the lower limits of detection for the Illumina MiSeq (687) and Sanger sequencing (682), respectively. Additionally, the selection of 1% for dichotomizing HIV-1 deep sequence data has also been empirically validated in a recent whole-genome deep sequencing study of HIV-1 (733) and employed in a genome-wide association study of HIV-1 drug resistance (734).

Our SVM analyses identified a cluster of amino acid polymorphisms associated with RAL failure in the α helix domain, including the mutations I203M and I208L. With this new information, we could map the majority of RAL failure samples to four largely independent mutational pathways characterized respectively by I203M, I208L, Q148/R, and N155H (Fig. 29). I203M has previously appeared on lists of INSTI resistance mutations in the earlier literature (735,736)[85–87]. Furthermore, I203M was previously characterized as a minor or accessory mutation with no evidence of directly reducing susceptibility, and has also been characterized as a natural polymorphism that is observed a substantial frequencies in un- treated individuals (729). In contrast, we have found almost no previous mention of I208L in the HIV-1 drug resistance literature.

We subsequently introduced the I203M, I208L mutations through site-directed mutagenesis, and used patient-derived viruses carrying I208L or I203M and I208L to evaluate susceptibility of these mutants to INSTIs *in vitro*. Our results indicate that these mutations in IN do not confer resistance to RAL or DTG (Fig. 30). However, these mutations could act as secondary mutations to mutations outside HIV-1 integrase gene. Resistance mutations in the HIV-1 3' polypurine tract, a conserved motif that primes the synthesis of plus-strand HIV-1 DNA during reverse transcription, have been shown to significantly reduce susceptibility of HIV to INSTIs in vitro (737). In addition, recent studies suggest that mutations conferring INSTI resistance may co-evolve with the upstream end of U3 and the downstream end of the U5, i.e. the conserved target sequence for dinucleotide cleavage. We have recently sequenced the LTR of the same Uganda patients failing INSTIs to determine possible linkage with the U5 and U3 target sequences with I203M and I208L. Although there is no evidence to date, there is also the possibility that IN mutations selected by INSTIs may also impact the efficient of integration into the cellular DNA by way of enhanced chromosomal DNA cleavage. We now have preliminary data suggesting that treatment failure with INSTI results in dramatic changed in the targeting of proviral DNA integration. How this is associated with the selected IN mutations associated with INSTI (as defined by this SVM analyses) is the subject of further study. Finally, resistance to a drug can also be manifested by increased replicative fitness even if susceptibility to drug is unaltered. For example, a 10-fold inhibition can be overcome in terms of viral burden if HIV-1 I203M or I208L replicates 10X more efficiently than wild type. However, our studies indicate that HIV-1 with I203M or I208L are less fit than the wild type HIV-1.

Further experimental work in our laboratory is now directed towards detecting mutations outside HIV-1 integrase gene and the integration patterns in the host genome. We suspect that that I203M and I208L are associated with INSTI resistance since these mutations define distinct evolutionary pathways emerging during INSTI treatment and failure to this treatment. This I203M and I208L evolutionary pathway emerged in lieu of N115H, Y143H/R, and Q148K/R pathways that definitely confer RAL resistance.

Chapter 7

7 The slow roll out of second generation INSTIs in Uganda and other LIMCs in the face of emergence resistance to Raltegravir

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7.1 Preface

In this Discussion chapter, I will first outline the slow roll out and need of second generation of INSTIs in low-to-middle income countries like Uganda. Part of this chapter is published in the journal, Infectious Diseases of Poverty (2019) but aspect of this topic were also addressed by the commentary published in Journal of Infectious Diseases (2017). For the article published in Infectious Diseases of Poverty, I wrote and co-authored the article with Dr. Arts. The article published in J. Infectious Diseases of Poverty was later presented as a featured article on journal's homepage.

In 2014, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and partners launched the 90-90-90 targets. The 90-90-90 global targets by UNAIDS calls for 90% of all people living with human immunodeficiency virus (HIV) to know their status, 90% of all people diagnosed with HIV infection to be on combined antiretroviral therapy (cART) and 90% of all people receiving cART to have suppressed the virus by 2020 (738). The world is progressing towards achieving these targets with most European countries close to 90-90-90 targets (739). Some countries like Denmark, Iceland, Sweden, Singapore, and the United Kingdom have already achieved these target while good progress towards the 90-90-90 targets are observed in Eastern and Southern Africa, Eastern Europe and central Asia (740) while countries in West and Central Africa are lagging farther behind.

In a recent report by UNAIDS, antiretroviral therapy (ART) was accessible to only 26% of children and 41% adults in Western and Central Africa, compared to 59% of children and 66% adults who had access to ART in Eastern and Southern Africa in 2017. There was also almost 50% reduction in number of AIDS related deaths in West and Central Africa compared to Eastern and Southern Africa (24% vs 42% respectively) (741). LICs carry 90% of global HIV burden, and though there has been good progress to achieve 90-90-90 targets, pretreatment drug resistance (PDR), non-adherence, and side effects associated with the current cART pose a great threat. PDR and transmitted drug resistance (TDR) are on the rise in low-income countries (LICs) (524,742) and the trend is not likely to change as countries implement World Health Organisation (WHO) “Treat All” recommendation (743). It has been shown that increase of TDR results in increased treatment switches (744) and positively correlates with cART roll out.

However, with limited treatment options, LIMCs are facing a dilemma of keeping patients on treatment regimens that are deemed less optimal in high income countries. Lack of access to drugs having higher genetic barrier to resistance contributes to the transmission of HIV resistant virus, and limited virological monitoring affects early detection of drug resistant mutations (DRMs). In LIMCs, first line (FL) cART commonly consists of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitors (NNRTIs) commonly efavirenz (EFV) or nevirapine (NVP), with Lopinavir/Ritonavir (LPV/r) or Atazanavir/Ritonavir (ATV/r) replacing NNRTI in second line (SL) therapy. More potent

drugs, Darunavir (DRV), Raltegravir (RAL), and Etravirine (ETR) are sparingly used and commonly assessed by patients on salvage therapy and only in a few treatment centres.

Routine viral load monitoring, more potent drugs with fixed dose combinations with higher genetic barrier to DR and adherence support are among areas of emphasis for rapid scale up and better care of patients (745). However, these guidelines poor implementation in LMICs due to lack of access to such drugs. It has been predicted that with no change of current regimens and TDR increasing beyond 10%, there will be 890 000 new deaths, 450 000 new infections and increase of cART cost of USD 6.5 billion by 2030 (746). I will discuss cART-based reasons underlying these challenges and mitigation strategies.

The low genetic barrier to drug resistance

The commonly used NNRTIs in FL, (NVP/EFV), and (LPV/r) based SL therapy in LMICs, have low genetic barrier to drug resistance compared to second generation NNRTIs, rilpivirine (RPV) and ETR. High-level resistance between these drugs has been reported (513) which reduces treatment response more so in patients with TDR. In Uganda for example, where NFV and EFV are frequently used in first line, over 96% FL failures, 75% SL failures, and 49% RAL failures, have NNRTIs resistance (540). In LMICs, over two-thirds of patients retain NNRTIs resistance even many years on second line therapy (540), and Steegen et al. show that ≥ 65% of second line failures had NNRTI mutation (747). Lack of HIV genotypic tests in most countries impedes early detection of drug resistant variants. Already HIV drug resistance (HIVDR) in patients failing LPV/r-based regimen has increased in Africa (748), and the number of patients who need second line therapy is likely to increase from 0.5–3.0 million in 2020 to 0.8–4.6 million by 2030. From 2012, Tenofovir (TDF) is the primary NRTI for first-line cART after replacing zidovudine and Stavudine. However, TDF resistance is on rise in LICs with 60% of patients who fail on TDF based cART having TDF resistance (749) and this could be due to lack of baseline resistance testing and prescription of TDF with EFV or NVP which have low genetic barrier to resistance.

Side effects of current regimens and poor adherence

Adherence to treatment is critical to a successful treatment response and may be influenced by cART. It reverses occurrence of mortality, cART related morbidity, hospital visits, and improves

immunological benefit of using ART. Improved adherence correlates with increased CD4 count (750) and is the second-best predictor of disease progression (751). Poor adherence may be associated with development of DRMs which may contribute to virological failure (VF). Studies have shown that contrary to the belief that people in LICs are naturally non-adherent to treatment, adherence can also be achieved in LICs (752).

The available cART in LICs is complex and associated with huge pill burden, short, and long-term medication side effects. Adherence was reported to be around 40% in a recent adolescent study in 23 sub-Saharan Africa countries (753). The study shows non-adherence as a key problem facing health care service in this region, and it is worsened by counselling services focusing on outcomes of non-adherence not causes. Short message service and treatment supporters can improve adherence in Africa (754), however, such strategies should go in hand with availability of more potent drugs with better safety and tolerability profiles.

Though LPV/r is associated with more adverse gastrointestinal effects than ATV/r or darunavir/ritonavir (755), it still forms the backbone of majority of second line treatment. It has recently been shown to increase cardiovascular risks of myocardial infarction and stroke in HIV infected patients in the US when compared to ATV/r (756). Boosted lopinavir and 2NRTI combination has been removed from other regimens category because of huge pill burden and greater toxicity (//aidsinfo.nih.gov/guidelines). HIVDR resulting in virologic failure during second line treatments in LICs, has been attributed to poor adherence other than LPV/r activity (615). In addition, the commonly used NNRTI, EFV, is associated with more adverse gastrointestinal effects and rashes compared to RPV (757).

Pretreatment HIV-1 drug resistance

Globally, over 10.1% of HIV infected patients have baseline DR (758) and it is associated with reduced treatment response in both HICs (759) and in LICs (760). Whereas prevalence of TDR remained stable in HICs 2002–2010 at 8%, in LICs, there has been an increase in prevalence with roll out of cART. In some countries, frequencies of NNRTI and NRTI DRMs in patients initiating cART increased from 0% (2006–2007) (570), to 8.6% (2009– 2010) (571) and 15.4% (2014–2016) (524). Despite an estimated prevalence of HIVDR of (7.4%) eight years after roll-out of cART, the estimated annual increase of PDR in East Africa is 29% and 14% in South Africa and

largely driven by high NNRTIs resistance (524). This confirms observed positive correlation between cART roll out and increase of TDR in LICs (761). In a national survey by 11 LICs countries on PDR, six out of 11 countries had prevalence of 10% and above which calls for change of first line regimen as per WHO guidelines on HIVDR (524). TDR in LICs is more common in NNRTIs (4.5%) and NRTIs (4%) than in protease inhibitors (2.8%) (760) unlike in Europe with less baseline NNRTIs resistance (2.5–2.9%) (762,763). Baseline NNRTIs resistance has been shown to cause more impact to treatment response (763). In LICs, prevalence of TDR in children ≤ 12 years was found at 42.7% in those exposed to prevention of mother to child transmission and 12.7% in unexposed (615). These countries now face the dilemma of overcoming TDR and this is further complicated by lack of virological monitoring and inaccessible genotyping test. Access to baseline genotyping test in the START trial (Strategic Timing of Antiretroviral Treatment trial) was found to be 0.1% in Africa, 1.8% in South America and 22% in Asia (22%) compared to over 80% in Europe (86.7%), United States (81.3%) and Australia (89.9%) (758). Moreover, Sanger sequencing commonly used in these countries cannot detect resistant variants below 20% and yet these variants been associated with treatment failure (732). Only 22% of patients on cART in middle and LICs get access to virological monitoring (764). This implies reliance on clinical and immunological monitoring which detect treatment failure late and this leads to emergence of more complicated DRMs. In children below three years, the impact of TDR is even stronger than in adults with odds ratio for failure of 15.3 and has been associated with VF and acquired DR (574).

Strategies to address challenges associated with current cART and ways to make more potent ART accessible in LICs

The most ART associated challenges in LICs are mainly rooted on HIV drug resistance among other factors like the scarcity of treatment options for HIV infected patients in LICs more so on those failing salvage therapy. Several recommendations can be made on how to address these challenges and improve the lives of people living with HIV in LICs.

Firstly, patent licences should be closely monitored, and local drug manufacturers be encouraged. Drug price is a major factor contributing to lack of access to these drugs especially due to patent restrictions. Third line therapy may be 18 times and seven times more expensive than FL and SL

treatment (765). However, with expired patent licenses and some expiring soon, it's high time for stake holders to advocate for voluntary and compulsory licensing among other strategies to make these drugs more affordable. Basing on data on active pharmaceutical ingredients exported in and out of India, the cost of treatment of HIV could be as low as USD 90 annually if substantial generic competition is enforced (766).

HIV integrase inhibitor, DTG has shown superior genetic barrier to resistance 185 and potency in patients with DRMs to RAL and elvitegravir. Once daily dosage of DTG can be given to patients initiating cART and those failing RAL-based cART as once or twice daily dosage depending on presence of integrase strand transfer inhibitors (INSTIs) mutations more so Q148K/R/H. National Institutes of Health consultation recommends the use of tenofovir alafenamide fumarate (TAF) which has less bone and kidney toxicity, and DTG, which may help reduce drug resistance and improve adherence in LICs (767). The compound patent for DTG is expected to expire in 2026 and with licenses on adults and pediatric formulations available to all LICs through Medicines Patent Pool and ViiV Healthcare, market competition of local manufacturers will likely increase access of drug in this setting. The initiative by International drug purchase facility (UNITAID) to enrol DTG in some LICs of Kenya, Uganda and Nigeria (768) should be encouraged. Neural tube birth defects in children born to mothers who were exposed to DTG during pregnancy still raises concern to the safety of using DTG in pregnant mothers. In a study that compared the birth outcomes between 1729 pregnant women on DTG based ART and 4359 mothers on EFV in Botswana, found no significant differences in the individual outcomes of stillbirth, neonatal death, preterm birth, very preterm birth, small for gestational age, or very small gestational age, and severe side effects between patients on DTG or EFV based ART (769). In a recent study looking at neural tube defects with DTG treatment in women who started DTG from the time of conception, 426 (0.94%) infants born from mothers who were initiated on DTG at conception, had neural birth defects of encephalocele, myelomeningocele and iniencephaly compared to 11 300 (0.12%) infants born to mothers on non-DTG regimen at conception (643). In same study, 2812 infants 208 who were born to mothers who initiated DTG during pregnancy none had neural tube defects. These observations suggest DTG associated neural tube defects may be dictated by the time of DTG initiation to pregnant mothers. EFV based combinations may be the best choice for HIV infected pregnant

women initiating ART but with potential reduction in use of NNRTIs based therapy in LICs, more research is necessary in area of DTG use and pregnancy. The current statement of WHO on potential risk of birth defects to infants born of mothers exposed to DTG at a time of inception, is to initiate pregnant women to EFV based regimen which has confirmed efficacy and safety profiles. DTG can only be used in childbearing women only when consistent contraception is guaranteed and where other first line regimens can't be used (770). To concur with this statement, a recent study carried across 13 European countries and Thailand investigating association of initiating EFV based ART during conception or first trimester of pregnancy and birth defects, found no significant difference in prevalence of birth defects between EFV based and non-EFV ART based groups (771). There are also reports linking the use of DTG to abnormal weight gain. In a study assessing weight change in patients switching from EFV/3TC/ emitricabine (FTC) to an INSTI-based regimen, greatest weight gain was observed in patients switched to abacavir/3TC/DTG combination (772). Another study reviewing patient data from observational SCOLTA project which was looking for drug related adverse effects in patients who started a regimen containing, DTG, RAL, EVG, DRV or ETR, found no difference in body mass index between patients on INSTIs-based ART and those on non-INSTIs regimens. However, precautions should be taken when drawing conclusions as these were retrospective observational studies. Therefore randomised, controlled studies are still needed to validate these observations.

ETR is largely patented in developing countries and with patent restrictions in leading manufacturing nations of India, China and Brazil, no generic form is available. The patent on novel compound expires in 2026 which is far beyond 2020 target and therefore more advocacies required. DRV has high genetic barrier to resistance and is effective in patients with multidrug resistant viruses (773) and was approved for use in treatment naïve adults in the US and European Union. No high-level resistance to DRV was observed in national survey of 350 patients failing second line therapy in South Africa [13]. At least three DRV associated mutations in combination with multiple protease inhibitor associated mutations are necessary for DRV resistance to occur (774) as shown in POWER 1 and 2 studies. In Madrid study of 1364 genotypes of cART naïve and experienced patients for the impact of HIV subtype to DRV and tipranavir, all 29 non-subtype B cART naïve patients had 100% susceptibility with DRV, and associated DRMs were more common in HIV subtype B virus than in non-subtype B viruses ($P < 0.001$) (775). In context that

majority of patients in LICs fail treatment with multiple DRMs, DRV provides best alternative for those switching to second line therapy. Despite compound patent on DRV expiring in 2013 and patents on pseudopolymorph and/ or on the combination with ritonavir been granted to most LICs, it is still not widely available in LICs. Countries should take advantage of availability of license not to enforce patents on DRV in sub-Saharan Africa and least developed countries to encourage wide manufacturing of generic forms to increase access.

Secondly, access to single pill formulations, drugs with high genetic barrier to resistance, and those with promising results in clinical trials, should be made a priority. Indeed, high TDR prevalence and lack of baseline resistance testing call for robust ART for patients starting on treatment. RPV approved for treatment of NNRTIs-naïve patients is more tolerable and allows simplification due to its single pill formulations of TDF/FTC/RPV, and TDF/TAF/RPV which improves adherence and general response to treatment. ETR is approved for use in NNRTIs experienced patients and has shown high potency to both wild type and NNRTIs resistant virus (776). However, the high HIVDR due to Y181C after NVP exposure (777) necessitates genotypic test before use of ETR as salvage therapy. Doravirine being tested in once daily doravirine/3TC/TDF combination, is more tolerable, has high efficacy, and activity against viruses with resistant mutations K103N and Y181C (778). It was non-inferior to EFV with 84% vs 81% of patients in doravirine and EFV arms achieving undetectable VL after 48 weeks respectively in phase 3 DRIVE-AHEAD study (779). Cabotegravir in advanced stages of clinical trials, look promising for its use as long acting injectable with monthly or bimonthly administration for pre-exposure prophylaxis use and treatment of HIV infection. It has shown better safety profile and high acceptability in low risk uninfected participants in on-going clinical trial HPTN 077 (780). Though there is possibility of cross resistance with already approved INSTIs through Q148 pathway, it still provides better alternative. In addition to minimal side effects, injectable cabotegravir provides convenience, flexibility, and fitness of patient lifestyles which improves adherence. Bictegravir when given in a fixed-dose combination with FTC and TAF, was not inferior to DTG given in DTG/FTC/TAF combination in treatment-naïve patients after 48 weeks in phase 3 of Study 1490 (781). MK-8591 a long-acting nucleoside reverse transcriptase translocation inhibitor has shown to be a promising long duration treatment and

prophylaxis in phase 1b clinical study showing half-life of 2.3 to 56.8 and 78.5 to 128 hours with patent and triphosphate form (MK-8591-TP) respectively (782).

Thirdly, HIV drug resistance surveillance should be emphasized as HIV drug resistance is one of indicators of ART program. The impact of HIVDR has been attributed to be 15.6% of AIDS deaths, 9.4% of new infections and 7.9% of ART costs from 2017 to 2021 (783). Therefore, monitoring of all three forms of HIVDR, TDR, and PDR, and acquired drug resistance, by putting in place checkpoints to track emerging and spreading HIVDR becomes very paramount. This becomes even more crucial at a time when countries are starting all persons infected with HIV on treatment and wide coverage of prevention of mother to child transmission programs. Much as prevention of mother to child transmission program has drastically reduced the number of babies acquiring HIV from their HIV positive mothers to < 10%, HIVDR especially to NNRTIs has been increasing in children who still become HIV infected through prevention of mother to child transmission program (Table 13). This is mostly attributed to initiating these children to first line treatment with NNRTIs component due to lack of recommended protease inhibitors in these settings. This therefore calls for more support to ART programs to ensure adequate and reliable supply of child dosage combinations in these countries. It is not uncommon in some of these settings for clinics to provide children formulations to adults by prescribing high dosage where there is shortage of adult regimens. This form of practice affects pediatric HIV treatment programs and may lead to treatment failure in adults.

Table 14. Prevalence of HIV pretreatment drug resistance in pediatrics and adults in low-income countries

	Type of population	Year	Region	HIV PDR to NNRTIs	Yearly increase in HIV PDR	Reference
a)	Children and infants:					
	PMTCT exposed	2016	Sub saharan Africa	32.4% (95% CL; 18.7-46.1%)	26.8% between 2004-2013	(615)
	PMTCT unexposed	2016	Sub saharan Africa	9.7% (95% CL; 4.6-14.8%)	-	(615)
	PMTCT exposed	2012-2013	Togo	81.80%	-	(776)
	PMTCT exposed	2007-2014	Zambia		21.5%-40.2% between 2007/2009-2014	(777)
	PMTCT exposed	2011-2014	South Africa	54.90%	-	(778)
	PMTCT exposed	2011-2014	Mozambique	59.20%	-	(778)
	PMTCT exposed	2011-2014	Swaziland	41.20%	-	(778)
	PMTCT exposed	2011-2014	Uganda	38.80%	-	(778)
	PMTCT exposed	2011-2014	Zimbabwe	74.70%	-	(778)
	PMTCT exposed	2010-2013	South Africa	52.00%	-	(779)
	PMTCT exposed	2011	South Africa	56.80%	-	[60]
	PMTCT unexposed	2010-2011	Uganda	7.50%	-	(573)
b)	All					
	All	2001-2016	South africa	11% (7.5-15.9)	23% (16-19)	(734)
	All	2001-2016	East africa	10.1% (5.1-19.4)	17% (5-30)	(734)
	All	2001-2016	West and central africa	7.2% (2.9-16.5)	17% (6-29)	(734)
	All	2001-2016	Latin and caribbean	9.4% (6.6-13.2)	11% (5-18)	(734)
	All	2016	South africa	11%	23%	(525)
	All	2016	East africa	15.50%	29%	(525)
	All	2016	West and central africa	7.20%	17%	(525)
	All	2016	Latin and caribbean	15%	15%	(525)
	All	2016	Asia		11%	(525)
	All	2000-2016	South Africa	8.50%	1.2-fold increase (95% CL; 1.13-1.23)	(780)
	Type of population		Region	levels of any HIV PDR	Yearly increase in HIV PDR	reference
	All					
	All	2008-2010	Angola	16%	-	(781)
	All	2016	Argentina	13%	-	(525)
	All	2012-2014	Bostwana	10%	-	(782)
	All	2003, 2007-2011	Cuba	22%	-	(783)
	All	2015	Mexico	15%	-	(784)
	All	2013-2014	Papua New Guinea	6%	-	(785)
	All	2000-2016	South Africa	10%	1.1- fold increase (95% CL; 1.06-1.15)	(780)
	All	2004-2014	Global	6.9% in 2010	9% in 2012	(786)

Abbreviations: CI: Confidence interval, HIV Human immune deficiency syndrome, PDR Pretreatment drug resistance, NNRTIs Non-nucleoside reverse transcriptase inhibitors, PMTCT Prevention of mothers to child transmission. -: not applicable

Much as the global burden of HIVDR especially in LICs is known and mitigation strategies well stipulated, most of these countries are struggling to implement these strategies due to mostly inadequate resources and lack of political will by governments in some countries. As a result, there is insufficient monitoring of emerging drug resistance. The early warning indicators by WHO for drug resistance provide alternative way of monitoring for emerging drug resistance in this setting. They include offering optimal 300 treatment and according to the guidelines, checking for percentage of patients with loss of follow up after 12 months, looking at percentage of patients retained on ART at 12 months, patients with on time pill pick up/ clinic appointment, drug stock outs, and looking at patients under viral load monitoring and suppression [56]. Much as there are challenges in implementing this form of monitoring, it remains the feasible tool to combat the challenge of rising HIVDR in LICs.

The HIVDR monitoring programs cannot afford not to prioritise population at most risk; girls and women, men who have sex with other men, sex workers, drug users, and people in fishing communities; who face a lot of stigma and discrimination. Surveillance on access of HIV services to these vulnerable groups needs to be emphasized by ensuring that monitoring and evaluation systems for reporting on implementation are functional. The generic form of DTG has allowed a rapid roll out of this drug in most LICs. It is expected to change the landscape of HIVDR in LICs due to its high genetic barrier to resistance, better tolerability, and safety profiles basing on research commonly done in subtype B viruses. However, more research is still required to access its efficacy in non-B subtypes since DTG associated drug resistance appears to be HIV subtype specific. As such, there should be well articulated treatment guidelines and frequent surveillance of resistance to guide its proper use in ART naïve and highly treatment experienced patients in LICs.

Conclusions

The availability of more potent ART in LICs is of utmost importance if UNAIDS 2020 and 2030 goals are to be realized and sustained not only in HICs but also in LICs. Countries that have embraced these targets will have to provide treatment to the increasing numbers of newly infected

individuals expected to be 323 800 000 annually from 30 million in 2017 to 36.4 million in 2025 [37]. In countries which rolled out cART earlier, the prevalence of TDR increased by 12.3% in span of four years and will likely keep increasing. With increased drug switches which is associated with TDR, and acquired drug resistance common in LICs, more potent ART offer therapeutic and preventive incentives which will reduce treatment costs and increase drug options. In addition to making these drugs available, governments must address current loopholes through their jurisdictions as well as addressing lack of political commitment and poor policy decisions seen in some countries. It's also imperative to strengthen quality service delivery in terms of retention of patients to treatment, support for adherence to cART, patient follow up, and adequate drug stocks to help achieve a free AIDS generation.

Roll out of second generation of INSTIs should be in concert with screening for INSTI resistance in LIMCs

DTG is increasingly being rolled out in many LMICs though INSTIs still account to only about 1% of active first-line regimens in LMICs (698). Due to cost related factors, INSTIs are sparingly used in LMICs with RAL-based regimens been used in highly treatment experienced with multiple non-INSTIs mutations (628,699,700). HIVDR presents a huge challenge for future treatments to outcome. Over 10-15% of patients will fail on ART each year due to HIVDR in LMICs. HIVDR to INSTIs has been extensively analyzed and screened in subtype B HIV-1 which predominate infections of HICs. Contrary, HIVDR especially to INSTIs in non-B subtypes is significantly lacking despite 90% of HIV-1 infections caused by non-B HIV-1 subtypes. To understand the burden of HIVDR to INSTIs in non-B HIV-1 subtypes, we have screened for DRMs associated with INSTIs in a cohort of Ugandan patients attending HIV-1 testing and treatment services at Joint clinical research center (JCRC), Kampala, Uganda. Samples from a total of 417 ART naïve and patients receiving different lines of ART were tested for presence of INSTIs associated DRMs. In this cohort, INSTIs associated mutations were not observed in INSTIs naïve (n=366) but in 47% of patients failing on RAL-based regimen (see chapter one). Among the 47% with INSTIs DRMs, 4% (2/51) of patients (subtype A and D) harboring multiple primary INSTIs DRMs conferring resistance DTG, a unique observation in both LMICs and HICs. Nonetheless, DTG resistance was shown to be rare in LMICs which encourages its use especially in ART naïve patients. Since 2018, TDF/3TC/DTG is now available in Uganda and

most other LMICs. Basing on absence of DTG cross resistance in INSTIs naïve patients, DTG provides best option for patients initiating ART and those failing RAL-based regimen in absence of multiple INSTIs primary mutations.

INSTIs susceptibility/phenotypic studies have remained elusive in most LMICs including Uganda. In this settings, national treatment guidelines rely almost exclusively on predictions from HIV-1 resistance databases including International AIDS Society-USA Drug Resistance Mutations Group (784) and Stanford HIV-1 drug resistance database (607) and yet our cohort of patients had unique combination of DRMs not observed previously (421). INSTIs associated resistance has been shown to be subtype-specific with some mutations preferentially selected in HIV-1 types B compared to non-B subtypes; for instance, Q148 mutations are more frequent in HIV-1 subtype B compared to non-B subtypes (492). Therefore, phenotypic assays to ascertain the level of susceptibility of these patient viruses was critical to understanding INSTI resistance in Uganda.

We phenotypically tested eleven HIV-1-derived integrases from subtype A, D, and recombinant A/D infected patients (RAL-failures) for susceptibility to DTG, RAL and EVG. The viruses harboring single RAL/EVG primary INSTIs mutations (~50%) remained susceptible to DTG with fold-change in EC_{50} values nearly equivalent to that of wild-type. This may imply that in such patients failing RAL-based regimens with single RAL/EVG primary INSTIs mutations, twice daily DTG in combination with best possible NRTIs, may suppress the virus and improve treatment outcomes. From this analysis, we confirmed that subtype A with E138A/G140A/G163R/Q148R and subtype D with E138K/G140A/S147G/Q148K viruses on RAL-based regimen are highly resistant to all currently available INSTIs in LMICs. The complexity of these mutations and level of resistance, >100-fold DTG, and >1000-fold RAL and EVG has not been observed before neither in subtype B or non-B HIV-1 infected individuals. These results indicate that prescribing DTG-based combination to patients failing RAL-based regimens with multiple primary INSTIs DRMs may not be beneficial. Therapeutic strategy of keeping patients on regimens which sustains DRMs associated with reduced RC may help to avoid continual immune deterioration especially in settings with limited ART options. Interestingly, viruses carrying multiple INSTIs primary DRMs showed divergent viral fitness with E138K/G140A/S147G/Q148K showing severely impaired viral fitness and

E138A/G140A/G163R/Q148R having nearly wild type replicative capacity. To maintain active infection in patients with impaired replicative capacity, the loss could be compensated by emergence of secondary mutations which restore viral fitness and/ or even increase fitness in presence of INSTIs (671). It has also been shown that maintaining NRTI such as lamivudine (3TC) or emitricabine (FTC) in a treatment regimen even with the emergence of associated drug resistance helps to avoid increased viremia (785) and may even help to stabilize the FTC/3TC resistant mutation, M184V which has been associated with delayed emergence of mutations conferring resistance to DTG (455,785).

To further characterise INSTI resistance to different INSTIs in HIV-1 non-B subtypes, we tested 8 patient-derived viruses from our cohort for susceptibility to novel BIC and CAB INSTIs. Recombinant viruses with HIV-1 IN harboring single or in combination with secondary mutations remained susceptible to both BIC and CAB. Presence of multiple primary INSTIs DRMs leads to increased resistance to CAB and BIC. Overall, BIC showed more potency against viruses carrying various INSTIs-associated resistant mutations compared to CAB ($p=0.03$). Though not currently indicated, BIC and CAB offer alternative to patients with single primary and or in combination with secondary mutations especially in settings with limited HIV-1 drug options.

Over the years, next generation sequencing has increasingly been used in clinical monitoring of HIVDR especially in LMICs where treatment failure and interrupted therapy often results in primary drug resistant viruses fading in the patient's population below the limit of Sanger sequencing detection. The role of minority variants remains to be elucidated in HICs, but in Uganda, low frequency drug resistance variants may account for over 64% of HIV-1 patients failing first-line and where dominant drug resistant HIV-1 was not detected (732). In our screening study for INSTIs DRMs, we observed that over 50% of patients failing RAL-based regimens in Uganda lacked signature INSTIs DRMs, either as dominant in the intrapatient HIV-1 population or low frequency variants. Using deep-sequencing and SVM analysis, we identified I203M and I208L mutations in the HIV-1 IN coding region of subtype A and D infected patients failing RAL but we failed to confirm that these mutations conferred resistance to RAL and DTG. From structural analysis, I203 and I208 residues are found in C-terminal domain and quite distal from the HIV-1 IN active site. The I203M and I208L were not linked to any other IN mutations

or polymorphisms but new findings suggest that these mutations may have co-evolved with changes in the HIV-1 IN recognition sequence in the proviral DNA (upstream start of U3 and downstream end of U5). The combination of the I203M and/or I208L mutations with mutations in the U3 and U5 sequence may confer resistance to INSTIs by increasing the binding of viral or host DNA, increased dinucleotide cleavage, and/or increased integration efficiency. It is important to note that both I203M and/or I208L cause replication fitness loss in subtype A, B, or D IN coding region within an NL4-3 backbone but we had not yet sequenced and compared the U3 and U5 LTR sequences nor have we introduced the patient-specific, subtype-specific U3 and U5 sequences linked with the I203M and/or I208L IN sequences. We are currently exploring this possibility leading to INSTI resistance and that would confirm the high odds ratio that I203M and I208L are linked to INSTI resistance in about 1/2 to 1/3rd of the subtype A and D infected patients failing a RAL-based treatment regimen in Uganda.

In a nutshell, emergence of INSTIs resistance appears in part to be subtype specific with even N155H reducing DTG susceptibility more so in subtype A and D than in subtype B HIV-1. Surveillance for INSTIs-associated resistance remains critical in LMICs for early detection of resistance mutations. Early detection of virological failure, detection of novel INSTIs-DRMs, and increased HIVDR surveillance remains critical in LMICs to avoid the irreversible and detrimental effects of HIVDR.

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Appendix

High Time to Start Human Immunodeficiency Virus Type 1–Infected Patients on Integrase Inhibitors in Sub-Saharan Africa

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CORRESPONDENCE

High Time to Start Human Immunodeficiency Virus Type 1–Infected Patients on Integrase Inhibitors in Sub-Saharan Africa

Steegen and colleagues reported results from a national survey in South Africa aimed at determining the prevalence of antiretroviral drug resistance in patients who are not responding to protease inhibitor–based treatment [1]. They report at least 1 major mutation in 16.4% of 350 participants with no high-level resistance to darunavir/ritonavir (DRV/r) and low high-level resistance (5.2%) to etravirine (ETR). The number of patients who need second-line therapy are expected to rise from around 300 000 in 2013 to 2–4 million by 2030 [2]. The reduction in cost for first-line treatment in Africa has increased antiretroviral therapy (ART) rollout in many countries, with a "test and treat" strategy initiated in some countries such as Uganda. This will inevitably increase the number of people on ART, as well as the frequency of human immunodeficiency virus (HIV) drug resistance. Transmitted drug resistance has been shown to increase as more people are put on ART [3], and this is associated with virological failure. These factors together result in rising drug resistance and, therefore, more first-line failures. Such patients will require second-line therapy, which may be 2.4 times more expensive than first-line therapy [2]. Lopinavir/ritonavir has been commonly used due to its high genetic barrier compared with nonnucleoside reverse transcriptase inhibitors (NNRTIs) and its being able to be used in rifampicin-based tuberculosis treatment. However, because of toxicity and substantial cross-resistance with other protease inhibitors, it is no longer recommended [4]. Because atazanavir/ritonavir is available in a heat-stable, fixed- dose formulation and is less expensive than lopinavir/ritonavir [5], it is now commonly used as a second-line therapy.

Stegeen and colleagues highlighted the important observation of no high-level resistance to DRV/r and relatively low frequency resistance to ETR, though intermediate resistance was quite high (32.8%). This is encouraging as many patients failing on second-line therapy will eventually require third-line regimens. The recommended salvage therapy consists of raltegravir, DRV/r, and ETR. But the cost of salvage therapy is quite high. It may be 18 times and 7 times more expensive than first-line and second-line regimens, respectively [5]. Pretreatment drug resistance, which results in numerous drug switches [6], also leads to limited drug options. We have previously reported 18% drug-resistant mutations to ETR and rilpivirine in HIV subtype C–infected patients failing efavirenz- and nevirapine-based first-line therapy recruited in the Europe-Africa Research Network for Evaluation of Second line Therapy study. The striking observation in the study was high drug resistance of 40% to ETR and 51% to rilpivirine due to amino acid substitutions at Y181, H221, K101, and 138 in patients failing efavirenz [7].

Despite no NNRTI exposure at the time of HIV drug resistance testing for 347 participants in the Steegen et al study, 65% had ≥1 NNRTI mutation with K103N being the predominant one. Numerous studies have shown slow clearance of NNRTI-resistant variants in patients after exposure to efavirenz or nevirapine. Two-thirds of patients retain NNRTI mutations after many years on second-line therapy. Therefore, the success of salvage therapy will require the use of even higher genetic barrier drugs to mitigate the emergency of these resistant variants. It has been demonstrated that the majority of patients on salvage therapy can achieve viral suppression, but this comes at a cost of using more potent drugs, which are more expensive and not readily available. Indeed, there have been drug stockouts and patients going on drug holidays, resulting in viral rebound and treatment failure. Therefore, the challenge is sustainability of these patients on such medications.

The integrase strand transfer inhibitors (INSTIs), especially dolutegravir with a high genetic barrier, could be used as first-line regimens, as recommended by the World Health Organization [4]. Currently, no patient has developed resistant mutations against dolutegravir when used as first-line therapy [8]. It was superior to raltegravir when used in INSTI-naive patients [9]. Maximizing use of more potent drug options early in treatment before exposing the patient to limited and more expensive options of second and salvage therapy appears to be the most appropriate way of reducing HIV-1 drug resistance in low-income countries.

Note

Potential conflicts of interest. Author certifies no potential conflicts of interest. The author has submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Prior-Season Vaccination and Risk of Influenza During the 2014–2015 Season in the United States

To the Editor—The recent article by Skowronski and colleagues [1] raises the possibility that repeated influenza vaccination may increase one's likelihood of influenza illness during some seasons. During the 2014–2015 season in Canada, individuals reporting influenza vaccination in 3 consecutive seasons experienced an increased likelihood of influenza A(H3N2)-associated illness compared with those who reported no vaccination during the 2014–2015 season in Canada [2]. In contrast, individuals reporting vaccination during 2014–2015 but in neither of the 2 prior seasons had decreased risk of A(H3N2)-related illness compared to the consistently unvaccinated. The authors hypothesize that repeat vaccination with the same A(H3N2) vaccine component increased the likelihood of infection with antigenically drifted A(H3N2) viruses. However, similar conditions in the United States during the 2014–2015 season were not associated with increased risk of illness among individuals vaccinated in 2 consecutive seasons or with significant protection for those vaccinated only in 2014–2015 [3]. Differential vaccine effectiveness (VE) against multiple A(H3N2) clades that circulated during 2014–2015 complicates interpretation of subgroup analyses of VE by prior vaccination [4]. In addition, differences in ascertainment of prior-season vaccination status may contribute to some of the difference observed in the US and Canadian studies. For comparison, we conducted additional analyses of data from the US Influenza Vaccine Effectiveness (Flu VE) Network to investigate the effects of vaccination in 3 consecutive seasons on vaccine effectiveness against A(H3N2)-associated illness during the 2014–2015 season.

The US Flu VE Network conducts annual studies of VE using the test-negative study design that is also used in Canada. In the Canadian study, current and prior-season vaccination status is based on a combination of patient self-report and sentinel practitioner documentation. In the US Flu VE Network, current season vaccination status is also based on a combination of patient self-report and electronic immunization records; however, prior-season vaccination is based on immunization records only. Misclassification of vaccine history may result from inaccurate self-report or incomplete immunization records. One study that compared self-reported influenza vaccination to an immunization registry found that patients overreported vaccination by approximately 10% [5]; recall of prior seasons' vaccination may be less accurate. To minimize misclassification of vaccination history in 2 prior seasons, we considered documented doses only among patients aged ≥9 years with medical records available for at least 2 years prior to enrollment, and excluded patients who reported 2014–2015 influenza vaccination that was not documented. After adjusting for age and other potential confounding variables, we found no statistically significant association between vaccination in 3 consecutive seasons and A(H3N2)-related illness during 2014–2015 (Table 1). However, we observed the highest point estimate among persons vaccinated in 2014–2015 only. A sensitivity analysis restricted to the main genetic group (clade 3C.2a) of antigenically drifted A(H3N2) and influenza negatives resulted in similar estimates (data not shown). Although the higher point estimate for vaccination only in 2014–2015 is consistent with potential negative interference from prior vaccination [1], our results do not support evidence of increased likelihood of influenza due to A(H3N2) viruses among repeatedly vaccinated individuals compared to those unvaccinated in 3 consecutive seasons.

The US Advisory Committee on Immunization Practices recommends annual vaccination for all persons aged ≥6 months to prevent influenza and its complications. This policy results in annual revaccination among a substantial part of the population receiving influenza vaccines. In previous seasons, the US Flu VE Network has reported modest effects of repeated vaccination on vaccine effectiveness [6–8], similar to findings for 2 of 3 A(H3N2) seasons in the Canadian study [1]. Analyses for 2014–2015 from Canada are the first to report statistically significantly increased likelihood of influenza A(H3N2) among revaccinated individuals compared to unvaccinated, although one other study reported increased likelihood of influenza A(H3N2)-related illness among persons vaccinated in 2014–2015 in a population with a large percentage of repeat vaccination [9]. Similar to the United States, a multicountry European study reported higher VE among persons vaccinated only in 2014–2015 without prior-season vaccination, but observed no increased likelihood of influenza A(H3N2)-related illness among those vaccinated both seasons compared to those not vaccinated [10]. As Skowronski and colleagues point out, underlying differences between the

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Bsc 2006-2009

Honors and Awards:

Province of Ontario Graduate Scholarship
Schulich School of Medicine and Dentistry, Western University
2019-2020

Queen Elizabeth II Diamond Jubilee scholarship
Schulich School of Medicine and Dentistry, Western University
2016-2018

Dr. Frederick W. Luney Graduate travel award
Schulich School of Medicine and Dentistry, Western University
2018 and 2019

Western Graduate Research Scholarship
Schulich School of Medicine and Dentistry, Western University
2016-2020

Towards an HIV Cure: Research-for-Cure Academy fellow
International AIDS Society (IAS)
2019

17th European AIDS Conference- Complementary registration
European AIDS Clinical Society (EACS)
2019

28th International Workshop on HIV Drug Resistance and Treatment Strategies- Complementary registration
Southern African HIV Clinicians Society
2019

26th International Workshop on HIV Drug Resistance and Treatment Strategies. Best poster award
Southern African HIV Clinicians Society
2017

Eminent QEII Scholars award from Lieutenant Governor of Ontario Her Honour the Honourable Elizabeth Dowdeswell
Western University
2019

Advanced Virologic Laboratory Training award
Case Western Reserve University
2015

Work experience

Research Assistant
Center for AIDS Research (CFAR) Laboratories at Joint clinical Research Centre (JCRC), Kampala, Uganda
2011-2016

Teaching and training

Teaching Assistant
Western University
2019-2020

Language and cultural instructor, Global Health Systems in Africa, MMAsc program
Western University
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Training and mentoring of undergraduates
Western University
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Training and mentoring of undergraduates
Center for AIDS Research (CFAR) Laboratories, Joint clinical research center, Kampala, Uganda
2011-2016

Community involvement

Presentation on HIV treatment in Low resource countries for Schulich's Global Health community health night
Western University
2019

Pre-departure session to first year medical students going to Uganda for placement through internalization program
Western University
2019

A judge for Scinapse Undergraduate Science Case Competition
Western University
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Reviewer of international peer-reviewed journals

Publications:

Ndashimye E, Arts EJ. Dolutegravir response in antiretroviral therapy naïve and experienced patients with M184V/I: Impact in low-and middle-income settings. Int J Infect Dis. 2021 Apr;105:298-303. doi: 10.1016/j.ijid.2021.03.018.

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