FROM BEDSIDE TO BENCH AND BACK: FUTURE OPTIONS FOR ANTIRETROVIRAL DRUGS IN NON-B HIV-1 SUBTYPES

by
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Dissertation presented for the degree of
Doctor of Medical Virology in the Faculty of Medicine and Health Sciences at Stellenbosch University

This thesis is a result of the collaborative doctoral programme between Karolinska Institutet, Sweden and Stellenbosch University, South Africa

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December 2020

DECLARATION

By submitting this dissertation electronically, I declare that this dissertation includes three original publications (two of which I am first author and one that I am co-author) in peer reviewed journals. The development and writing of the papers were the principal responsibility of myself and my co-authors. A declaration is included in the dissertation indicating the nature and extent of the contributions of the co-authors. The articles referred to in this thesis maybe subjected to international copy right regulations. In addition, I further declare that this thesis is a result of the collaborative doctoral programme between Karolinska Institutet, Sweden and Stellenbosch University, South Africa. The thesis has been reformatted to suit the format required by Stellenbosch University and there are no substantial changes compared with the version submitted at Karolinska Institutet for the awarding of the doctoral degree.

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December 2020

ABSTRACT

Introduction: HIV-1 drug resistance remains a burden in low- and middle-income countries (LMIC). Regardless of the advances in antiretroviral (ARV) therapy, there is an increase in the trend of acquired and pre-treatment drug resistance mutations (DRM) in LMIC affected by HIV-1 non-B subtypes. The overall aim of this thesis is to understand the potential subtype-specific differences in viral fitness and drug susceptibility against the drugs that target different stages of viral replication that includes, protease-mediated cleavage and maturation (**Paper I),** reverse transcription (**Paper II**) and integration (**Paper III**)

Methods: The thesis presents cross-sectional drug resistance data from predominantly HIV-1 non-B subtypes. It also includes virological drug sensitivity and enzyme-based *in vitro* assay results of recombinant viruses derived from predominantly HIV-1 non-B and few HIV-1B infected patients. The following areas were investigated:

a) the role that HIV-1 subtype C (HIV-1C) *gag-p6* gene could play in response to protease Inhibitors PIs in the absence of known PI primary mutations (**Paper I**).
b) *Ex vivo* potency of the newer drug 4'-Ethynyl-2'-Fluoro-2' deoxyadenosine (EFdA), first and second generation non-nucleoside reverse transcriptase inhibitors (NNRTIs) and Tenofovir alafenamide(TAF) against diverse HIV-1 subtypes (**Paper III**)
c) *Ex vivo* potency of Integrase strand transfer inhibitors (INSTIs) against diverse HIV-1 subtypes as well as genotypic resistance data comparing INSTI naïve and INSTI-experienced patients

Results: In **paper I,** the study showed an increase in viral fitness for HIV-1C viruses carrying the PYxE insertion in *gag-p6* when compared to the wild-type (WT) HIV-1C viruses. Furthermore, some PYxE-carrying viruses had low sensitivity to LPV and (TAF) when tested in drug sensitivity assays. Clinical data analysis showed a higher pre-therapy viral load and a decrease in $CD4^+$ T-cell counts for patients harboring PYxE-carrying viruses when compared to WT. P**aper II**. demonstrated that EFdA has a high inhibitory potential independent of HIV-1 subtype and high antiviral activity against resistant viruses. However, HIV-1C viruses had a significantly reduced susceptibility to NNRTIs, specifically Rilpivirine and Etravirine. In **paper III,** the drug susceptibility of INSTIs results indicated that INSTIs such as Dolutegravir (DRV), Bictegravir (BIC) and Cabotegravir (CAB) inhibited non-B subtypes significantly as compared to HIV-1B subtypes.

Conclusion: Inferences can be made from the results to suggests that subtype-specific differences play an essential role in influencing the ARV susceptibility which could further

impact the treatment efficacy in sub-optimal adherence. To reduce the trend of increasing DRMs in non-B HIV-1 subtypes which are mainly dominating in LMICs, adherence support and viral load monitoring should be prioritized. A rapid adaptation of INSTIs and newer drugs that have long-acting potential is encouraged. However, pre-clinical studies and clinical trials that are mainly restricted to the HIV-1B enrolled patients, should be inclusive of non-HIV-1B infected patients before the massive roll-out of INSTIs and newer drugs continues in non-HIV-1B dominated settings.

OPSOMMING

Inleiding: weerstand teen HIV-1 is steeds 'n las in lande met lae en middelinkomste (LMIC). Ongeag die vooruitgang in antiretrovirale (ARV) terapie, is daar 'n toename in die neiging tot verkrygde en voorbehandelde geneesmiddelweerstandsmutasies (DRM) in LMIC wat beïnvloed word deur HIV-1 nie-B-subtipes. Die oorhoofse doel van hierdie tesis is om die potensiële subtipes-spesifieke verskille in virale fiksheid en geneesmiddelgevoeligheid te verstaan teenoor die medisyne wat verskillende fases van virale replikasie teiken, wat proteasemedieerde splyting en volwassenheid insluit (**Vraestel I**), omgekeerde transkripsie (**Vraestel II**) en integrasie (**Vraestel III**)

Metodes: Die tesis bied dwarsdeursnit-gegewensweerstandigheidsdata van hoofsaaklik HIV-1 nie-B-subtipes. Dit bevat ook virologiese sensitiwiteit vir die geneesmiddels en in vitro gebaseerde in-vitro-ondersoekresultate van rekombinante virusse afgelei van hoofsaaklik HIV-1 nie-B- en min HIV-1B-geïnfekteerde pasiënte. Die volgende gebiede is ondersoek:

a) die rol wat HIV-1 subtipe C (HIV-1C) gag-p6 geen kan speel in reaksie op protease-remmers PI's in die afwesigheid van bekende PI primêre mutasies **(Vraestel I**).
b) Die potensiaal van ex vivo van die nuwer geneesmiddel 4'-Ethynyl-2'-Fluoro-2 'deoxyadenosine (EFdA), eerste en tweede generasie omgekeerde transkriptase-remmers (NNRTI's) en Tenofovir alafenamide (TAF) teen diverse HIV-1 subtipes **(Vraestel II**)
c) Ex vivo-sterkte van Integrase-streng-oordrag-remmers (INSTI's) teen diverse HIV-1 subtipes, asook genotipiese weerstandsdata wat INSTI-naïef en INSTI-ervare pasiënte vergelyk. (**Vraestel III**)

Resultate: In **vraestel** I het die studie 'n toename in die virale fiksheid vir HIV-1C-virusse wat die PYxE-invoeging in *gag-p6* dra, vergelyk met die wilde-tipe (WT) HIV-1C-virusse. Verder het sommige PYxE-draende virusse 'n lae sensitiwiteit vir LPV en (TAF) gehad toe hulle in medisyne-senisitiwiteitsondersoeke getoets is. Kliniese data-ontleding het getoon dat 'n hoër virale lading voor die terapie en 'n afname in CD4 + T-seltellings was vir pasiënte wat PYxE-draende virusse bevat in vergelyking met WT. **Vraestel II**. aangetoon dat EFdA 'n hoë remmende potensiaal het, onafhanklik van HIV-1-subtipe en 'n hoë antivirale aktiwiteit teen weerstandige virusse. HIV-1C-virusse het egter 'n aansienlike verminderde vatbaarheid vir NNRTI's, spesifiek Rilpivirine en Etravirine, gehad. In **vraestel III** het die geneesmiddelgevoeligheid van INSTIs-resultate aangedui dat INSTI's soos Dolutegravir

(DRV), Bictegravir (BIC) en Cabotegravir (CAB) nie-B-subtipes beduidend belemmer het in vergelyking met die subtipes HIV-1B.

Gevolgtrekking: Uit die resultate kan afleidings gemaak word om aan te dui dat subtipespesifieke verskille 'n wesenlike rol speel in die beïnvloeding van die ARV-vatbaarheid, wat die effektiwiteit van die behandeling in sub-optimale nakoming verder kan beïnvloed. Om die neiging tot toenemende DRM's in nie-B HIV-1 subtipes te verminder, wat hoofsaaklik in LMIC's oorheers, moet aanhegtingsondersteuning en monitering van virale ladings geprioritiseer word. 'N Vinnige aanpassing van INSTI's en nuwer medisyne wat langwerkende potensiaal het, word aangemoedig. Voor-kliniese studies en kliniese toetse wat hoofsaaklik beperk is tot die HIV-1B-ingeskrewe pasiënte, moet egter nie-HIV-1B-geïnfekteerde pasiënte insluit voordat die massiewe implementering van INSTI's en nuwer medisyne in nie-HIV-1B voortduur. gedomineerde instellings

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all the people involved in the studies for my doctoral education at Karolinska Institutet (KI) and Stellenbosch University (SU) and this mainly includes the **Management and administrative staff at both SU and KI**, for my making my doctoral education possible. Although it was not an easy path, riddled with many challenges, the doctoral experience has brought me many reflections about the meanings and values of research as well as the dedication and patience required when working with diverse people in a dynamic and interactive environment.

I would like to extend my gratitude specifically to the following people:

- Associate professor **Ujjwal Neogi** and Professor **Susan Engelbrecht** as my main supervisors for my doctoral education at KI and SU, respectively, for their support, guidance, and encouragement. You have imparted wisdom in me that is immeasurable to compare and although you put a lot of pressure on me to work hard, as I can remember quite a few sleepless nights working, it was without reason and now I bear the fruit of your labor. Your scientific research experience and expertise are invaluable. I will forever be grateful and mindful of your supervision. Thank you.

- Professors **Anders Sönnerborg** and **Anna-Lena Spetz** as my co-supervisors from KI, for their support and encouragement, to think critically as a scientist and to develop the enthusiasm that is indispensable to the success of my doctoral education. Thank you.

- Dr Graeme Brendon Jacobs, for your unwavering support and guidance. You have been a great pillar of support that historically starts from when I was just a naïve, young honors student entering the halls of Stellenbosch University, and who was yet to envisage the meaning and value of HIV research. Words alone cannot express how I cherish your encouragement, your training in the Laboratory, and the wholeheartedness gestures you show that can only be translated as invaluable. You were not only my co-supervisor for my doctoral studies but also a dear friend that I hold in high regard. I will never forget your support. Thank you.

- Professors **Stefan Sarafianos** and **Kamalendra Singh** from Emory and Missouri Universities (USA) respectively for their collaborative support for my Ph.D. projects. Thank you.

- Professor **Wolfgang Preiser** from SU, my mentor, for your advice, support, and inspiration. Thank you.

- To all my friends and colleagues from the Division of Medical Virology at SU and with special mention to my ‘ Ph.D. comrade in arms’- **Given, Vurayai** and **Emmanuel –** thank you all for your encouragement.

- To the National Health and Laboratory service (NHLS) staff at Tygerberg, South Africa and with special mention to **Tania**, **Mathilda** and **Amanda** for their support over the years. Thank you.

- My colleagues and friends, both former and current at LabMed KI: **Hissa, Haleh, Giota, Flora, Ashwathy, Noelia, Ashok, Mira, Shilpa, Xi, Wang, Shambhu, Lydia, Rafa, Avinash Elisa, Shuba, Robert, Sara, Katie, Harpa, Emon, Anoop, and Maike –** Thank you all for your amazing company and support during my doctoral studies in Sweden!

Finally, it is said “family is the first essential cell of human society” -Pope John XXIII, and even more to me - the very fiber of my identity, foundation, inspiration and our motivation for living. My deepest and most sincere gratitude goes to my family – my mum (**Lydia**), sisters (**Dephne and Brandner**) and brother (**Dereck**) for you unrivaled support, encouragement that not only extends to my welfare but also an interest in my scientific projects. My outmost and passionate gratitude goes to my dear and loving wife **Emily**, for your support, and encouragement all through the years of my studies. It was not always easy dealing with a stressed husband, but you put up with me in the difficult times of my studies and lit the fire of motivation in me. My dear **Emily** you inspired me to realize the potential I have and were and always will be my partner, friend, confidante and soulmate. Thank you for your invaluable support.

DEDICATION

I dedicate this to my family, friends, and most importantly, to all the people that work for the betterment of people living with HIV.

39:4.13 – Urantia Book

“There is no material reward for righteous living, but there is profound satisfaction — consciousness of achievement — and this transcends any conceivable material reward.

LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. van Domselaar Robert*, **Njenda *T. Duncan****, Rao Rohit, Sönnerborg Ander, Singh Kamalendra, Neogi Ujjwal, (2019) *HIV-1 subtype 1 C with PYxE insertion has enhanced binding of Gag-p6 to host cell protein ALIX and increased replication fitness*
J. Virol. 93:e00077-19. DOI:10.1128/JVI.00077-19 [PMID: 3076057]

II. ***Njenda T. Duncan,*** Aralaggupe Shambhu, Singh, Kamalendra; Rao Rohit; Sönnerborg Anders, Sarafianos Stefan; Neogi Ujjwal, 2018. *Antiretroviral potency of 4'-Ethnyl-2'-Fluoro-2'-deoxyadenosine, Tenofovir alafenamide and second-generation non-nucleoside reverse-transcriptase inhibitors across diverse HIV-1 subtypes* J Antimicrob Chemother (2018); 73: 2721–2728 DOI: 10.1093/jac/dky256 [PMID: 30053052]

III. Neogi Ujjwal, Singh Kamalendra, Aralaguppe G. Shambhu, Rogers C. Leonard, ***Njenda T. Duncan***, Sarafianos G. Stefan, Hejdeman Bo and Sönnerborg Anders, (2018). *Ex-vivo antiretroviral potency of newer integrase strand transfer inhibitors cabotegravir and bictegravir in HIV type 1 non-B subtypes.* AIDS. 2018 Feb 20;32(4).
DOI: 10.1097/QAD.0000000000001726 [PMID: 29239896]

- ***Equal contribution**

LIST OF RELATED SCIENTIFIC PAPERS NOT INCLUDED IN THESIS

I. Mikasi SG, Gichana JO, Van der Walt C, Brado D, Obasa AE, **Njenda D**, Messembe M, Lyonga E, Assoumou O, Cloete R, Ikomey GM, Jacobs GB. *HIV-1 Integrase Diversity and Resistance-Associated Mutations and Polymorphisms Among Integrase Strand Transfer Inhibitor-Naive HIV-1 Patients from Cameroon*. AIDS Res Hum Retroviruses. 2020 Jan 13.
doi: 10.1089/AID.2019.0264 PubMed [PMID: 31830799]

II. Hill KJ, Rogers LC, **Njenda DT**, Burke DH, Sarafianos SG, Sönnerborg A, Neogi U,Singh K. *Strain-specific effect on biphasic DNA binding by HIV-1 integrase.*AIDS.2019 Mar 1;33(3):588-592.
doi: 10.1097/QAD.0000000000002078. PubMed [PMID:30475264];

III. Ikomey GM, Assoumou MCO, Gichana JO, **Njenda D**, Mikasi SG, Mesembe M, Lyonga E, Jacobs GB. *Observed HIV drug resistance associated mutations amongst naïve immunocompetent children in Yaoundé, Cameroon.* Germs. 2017 Dec 5;7(4):178-185. doi: 10.18683/germs.2017.1124. eCollection 2017 Dec.
PubMed [PMID: 29264355]

INTERNATIONAL PRESENTATIONS AT MEETINGS

- **EUResist - AREVIR meeting 2017, Cologne, Germany**

  Title: Potency of EFdA on diverse subtypes with or without prior resistance

  Presentation type: Oral Presentation

- **HIV and Hepatitis Nordic conference 2019, Stockholm, Sweden**

  Title: Investigating HIV-1 resistance patterns to protease inhibitors in HIV-1 infected patients failing second-line therapy in South Africa

  Presentation type: Poster Presentation

- **HIV and Hepatitis Nordic conference 2017, Stockholm, Sweden**

  Title: Potency of a novel translocation defective reverse transcriptase inhibitor, EFdA in comparison to non-nucleoside reverse transcriptase inhibitors on diverse HIV-1 subtypes

  Presentation type: Poster Presentation

- **HIV and Hepatitis Nordic conference 2016, Stockholm, Sweden**

  Title: Potency of a novel translocation defective reverse transcriptase inhibitor, EFdA in comparison to non-nucleoside reverse transcriptase inhibitors on diverse HIV-1 subtypes

  Presentation type: Poster Presentation

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LIST OF ABBREVIATIONS

3TC	Lamivudine
ADR	Acquired drug resistance
AIDS	Acquired Immunodeficiency Syndrome
ARV	Antiretroviral
BIC	Bictegravir
CA	Capsid
CAB	Cabotegravir
cART	Combination antiretroviral therapy
DNA	Deoxyribonucleic Acid
DRM	Drug Resistance Mutation
DSA	Drug sensitivity assay
DTG	Dolutegravir
EfdA	4'-Ethynyl 2-fluro deoxyadenosine
ENV	Envelope
EVG	Elvitegravir
FDA	Food and Drug Administration
FTC	Emtricitabine
GRT	Genotypic resistance testing
HIC	High income countries
HIV	Human Immunodeficiency Virus
HIV-1B	Human Immunodeficiency virus type 1 subtype B
HIV-1C	Human Immunodeficiency virus type 1 subtype C

HTS	High throughput sequencing
IN	Integrase
INSTI	Integrase strand transfer inhibitor
LMIC	Low and middle-income countries
LTR	Long terminal repeats
MA	Matrix
NC	Nucleocapsid
NOP	Naturally occurring polymorphism
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside analogue Reverse Transcriptase Inhibitor
PDR	Pre-treatment drug resistance
PI	Protease inhibitors
RAL	Raltegravir
RNA	Ribose nucleic Acid
RT	Reverse transcriptase
SS	Sanger sequencing
TAF	Tenofovir alafenamide
TMC125/ETR	Etravirine
WHO	World Health Organization

1 INTRODUCTION

Human immunodeficiency virus (HIV) drug resistance remains a challenge for the management of HIV-infected patients under combination antiretroviral therapy (cART) (1). Globally, Sub-Saharan Africa (SSA) is the region with the highest prevalence of HIV and with the most significant challenge in the management of HIV infections, as recently reported by the World Health Organization (WHO) (2). There has also been a substantial increase in the pre-treatment and acquired HIV-1 drug resistance rates across the 48 countries that comprise SSA. This chapter presents an overview of the replication cycle of HIV-1 and the different antiretroviral (ARV) drugs used to treat HIV-1. The end of the chapter describes a conspectus of the epidemiology and drug resistance situation in South Africa and Sweden as the two main countries from which HIV-1 patient material was used for the different studies mentioned in this thesis. The samples obtained from both countries represent some of the HIV-1 non-B subtypes (HIV-1B) that are a burden to SSA and globally.

1.1 HIV-1 REPLICATION CYCLE

HIV-1 is an RNA retrovirus approximately 100-120nm in diameter and targets host immune cells for its replication (3). Genetically, HIV-1 consists of three structural genes (gag, *pol, and env*); two non –structural regulatory genes (*tat, rev*) and four non-structural accessory genes (*vif, vpr, vpu, and nef)* (4-6). The three structural genes - *gag, pol* and *env* - encode the Gag capsid proteins, viral enzymes (Protease, Reverse Transcriptase and Integrase) and Env proteins respectively, that constitute the infectious viral particle (**Figure 1**)(7).

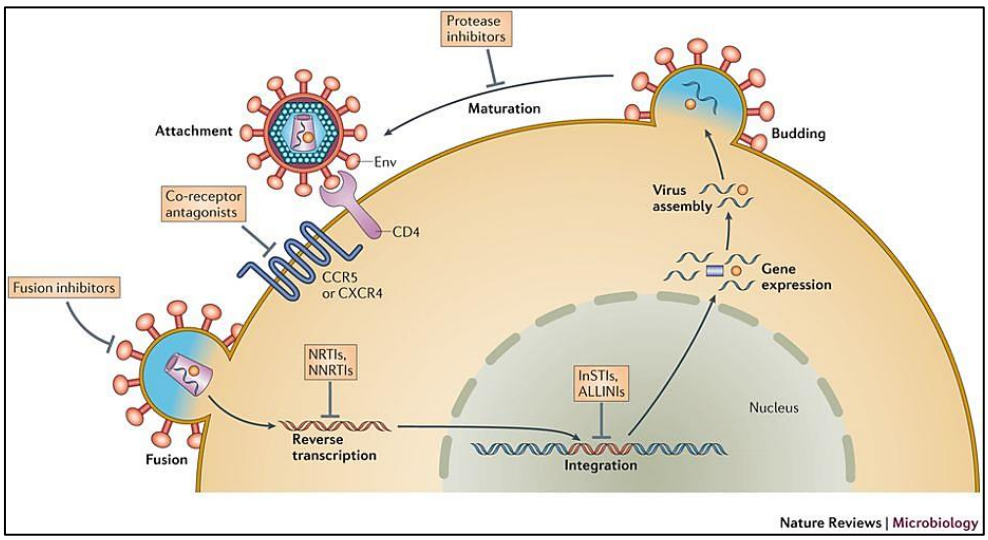

Figure 1. HIV replication cycle and drug targets. [Image reproduced with permission from Springer Nature and adapted from Nature Reviews (7)]

HIV transmission occurs via multiple routes that include heterosexual and homosexual intercourse, needle-stick injuries, transfusion with contaminated blood, and vertical transmission (Mother-to-child transmission- MTCT) (8-10). Upon an individual's infection with HIV, the replication cycle of the virus initiates when its envelope glycoproteins (gp120 and 41) facilitate its attachment to the CD4 receptor (primarily present on T- lymphocytes) (11) and the co-receptor (CCR5/ CXCR-4) (12) on the host cell membrane. Attachment of the virus leads to a fusion-dependent event with the host cell membrane that allows docking of the viral RNA genome into the cytoplasm (13). The viral RNA is then reverse transcribed into double-stranded complementary DNA (cDNA), and the cDNA then associates with host factors as well as the viral Integrase enzyme to form the pre-integration complex (PIC) (14). The PIC is then transported into the cell nucleus where the viral integrase enzyme enacts a strand transfer reaction that results in the integration of the viral genome into the host cell genome (15). The presence of the viral protein Tat (Transactivator of transcription) and host cell transcription factors lead to the expression of viral mRNA and after translation and post-translation modification events of the mRNA, viral proteins are then produced (16).

Viral proteins are then transported to the inner membrane via interaction with the endosomal sorting complex required for transport (ESCRT) machinery (17). The assembly of immature virions begins near the host cell inner membrane before they bud off and through cleavage events by the viral protease, emerge as infectious particles to enter the plasma or can be transmitted from cell to cell (18) to start another round of infection. In an untreated HIV infected patient, the number of viral particles released in a day could be as high as 10000 particles per cell (19). Sometimes the virus may remain transcriptionally silent after integration in the infected cell and establishes a latent reservoir (primarily in CD4 central and transitional memory T-cells) (20). HIV-1 latency is the reason why HIV-1 patients receiving ARV treatment cannot be completely cured (21).

1.2 HIV DRUG TARGETS AND ARV CLASSES

The main steps of HIV viral replication include binding and entry, reverse transcription, integration, viral assembly, and budding. These steps form the basis for the targets of the 6 different ARV drug classes (Nucleos(t)ide reverse transcriptase inhibitors (NRTIs), Non-Nucleoside reverse transcriptase inhibitors (NNRTIs), Protease inhibitors (PIs), Integrase strand transfer inhibitors (INSTIs) and entry inhibitors (sub-divided as fusion inhibitors (FI) and inhibitors of co-receptor usage), Table 1In total there are 31 United States of America (USA) Federal Drug Agency (FDA) approved HIV ARVs currently being marketed and the

brand names of their co-formulated products are also given below in **Figure 2** (22)

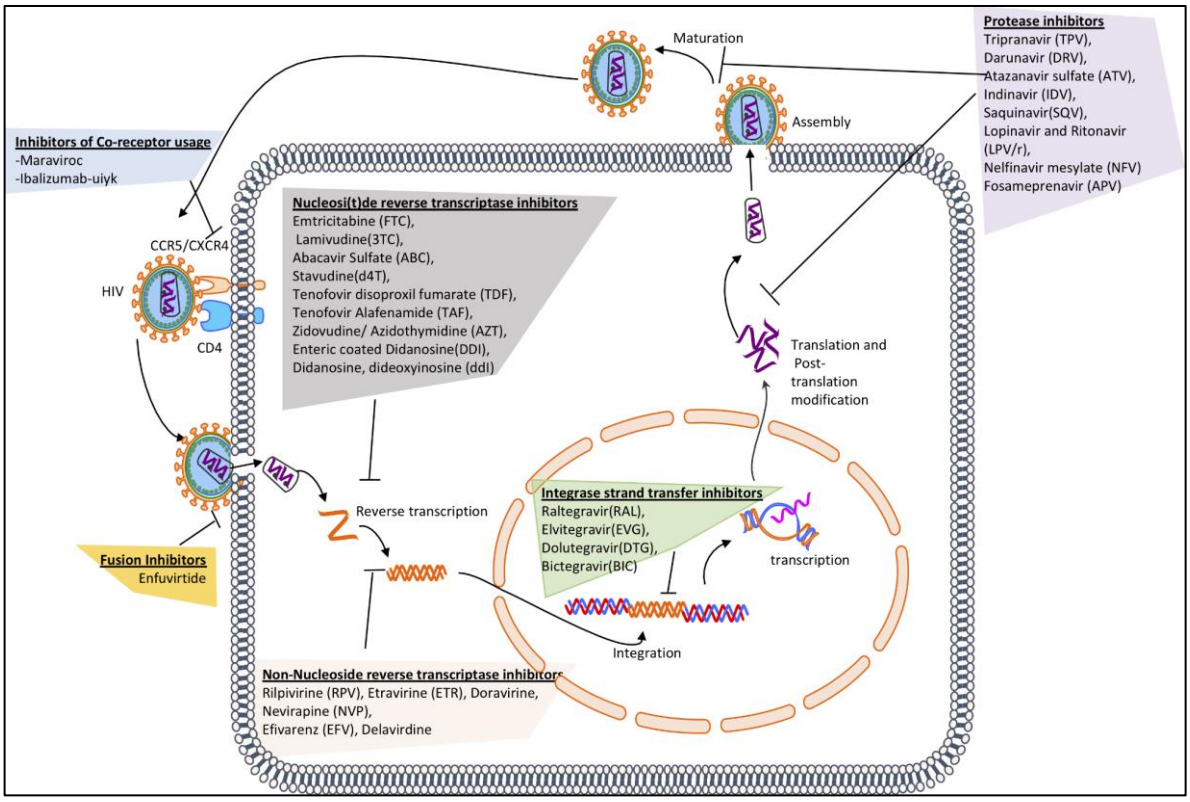

Figure 2. FDA approved antiretroviral drugs for HIV treatment shown to act on different stages of the HIV-1 replication cycle

Table 1. FDA approved Fixed-dose antiretroviral drugs for HIV currently marketed

Fixed-dose combinations	
Atripla (EFV+ FTC+TDF)	Prezcobix (DRV + Cobicistat)
Stribild (EVG + TDF + Cobicistat + FTC)	Trizivir (ABC +3TC +AZT)
Truvada (TDF + FTC)	Genvoya (EVG + cobicistat + FTC + TAF)
Triumeq (ABC + DTG + 3TC)	Biktarvy (BIC + FTC+TAF)
Complera (FTC + RPV + TDF)	Symtuza (DRV+ FTC +TAF + Cobicistat)
Combivir (3TC + AZT)	Dovato (DTG + 3TC)
Descovy (FTC + TAF)	Juluca (DTG + RPV)
Evotaz (ATV + Cobicistat)	Delstrigo (DOR + 3TC + TDF)
Epzicom (3TC + ABC)	Symfi (lo) (EFV +3TC +TDF)
Odefsey (FTC + TAF + RPV)	Cimduo (Temixys+ 3TC+TDF)

1.3 MECHANISM OF ACTION OF DIFFERENT ARVS

The classes of ARVs have different mechanisms of action that will be described below:

1.3.1 Nucleos(t)ide reverse transcriptase inhibitors (NRTIs)

NRTIs enter the cell by passive diffusion or are transported by a variety of carrier and ATP-dependent mediated membrane transporters. The most characterized membrane proteins for the transport of NRTIs are multidrug resistance proteins (MRPs) (23). Upon entering the cell cytoplasm, NRTIs are phosphorylated by the action of phosphotransferases and cellular kinases into triphosphate forms (24). NRTI triphosphate forms actively compete with the cell's natural dNTP substrates at HIV-1 RT's polymerase active site for incorporation into the growing cDNA strand synthesized by the HIV-1 RT enzyme. Once incorporated the triphosphate forms cause premature chain termination as they lack a 3'- OH group that would have facilitated the incorporation of the next dNTP substrate (25).

1.3.2 Non-Nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs do not have to be metabolized in the cell to gain activity against HIV-1. They gain cellular entry by passive diffusion as highly lipophilic molecules (26). They have been proposed to have three different mechanisms of action that express antiviral activity. The first classical mechanism is that of allosteric inhibition - NNRTIs bind in an HIV-RT pocket region that is 10Å away from the polymerase active site and cause a conformational change in HIV-RT affecting its ability to bind to the primer during reverse transcription (27).

The second mechanism (although controversial), involves inhibition effects on HIV-1 RT heterodimer formation. The inhibition restricts the enzyme's activity to simultaneous execute its polymerase and RNA degradation functions (26, 28). The third mechanism is believed to be an acceleration of cytoplasmic Gag-Pol processing by premature activation of the HIV-1 Protease enzyme. Premature activation of protease is hypothesized to occur when binding of NNRTIs to Gag-Pol proteins occurs, which then promotes oligomerization of the pol molecules by binding to the p66 subunit of HIV-RT. Premature cleavage of these Gag-Pol constructs restricts proteins required for viral particle assembly (26).

1.3.3 Protease Inhibitors (PIs)

PI cellular uptake can be via passive diffusion or mediated by Clathrin / Caveolae endocytic pathways (29). The tight binding interactions of PI on the dimer surface of the homodimer HIV-1 protease active site inhibits the enzyme's functioning to execute aspartic acid-mediated

cleavage of its substrates (30). The result of this inhibition affects the rate of viral assembly and can lead to the production of defective viral particles that are unable to establish another round of infection (31).

1.3.4 Integrase strand transfer inhibitors (INSTIs)

INSTI enter the cell by passive diffusion or endocytic adsorption. Once in the cytoplasm, they can bind to the integrase enzyme, but only access the enzyme's active site once there has been a conformational change that is only induced after the enzyme catalyzes the 3'-end processing reaction (32). The 3'-end processing reaction exposes a dinucleotide (CA) at the end of the viral cDNA that will eventually allow for integration into the host genome via the strand transfer reaction (32). To catalyze the strand transfer reaction in the nucleus, integrase requires association with the host factor - lens epithelium-derived growth factor (LEDGF/p75) (33) and divalent magnesium ions to stabilize its active site. The magnesium ions are sequestrated by INSTIs that have a β-diketo acid group as part of their chemical structure. The β-diketo acid group in INSTIs has a high binding affinity for magnesium ions (34).

1.3.5 Fusion Inhibitors (FIs)

Fusion inhibitor (FI) drugs are delivered subcutaneously and have an extracellular mechanism of action upon entering the systemic system (35). FIs are in the form of short synthetic polypeptides (36 amino acids in length) with a short half-life (less than 4 hours), and specifically, act as structural analogs that bind to the heptad region (HR-2) of HIV-1 gp41. HR-2 associates with HR-1 during hairpin formation to form the 6-helix bundle that facilitates attachment of the host cell and viral membranes (36). Hence, FIs competitively interrupt the hairpin formation process that facilitates HIV-1 *env* gp41 glycoprotein folding, preventing entry of the virus (36).

1.3.6 Inhibitors of co-receptor usage (CCR5 antagonists)

CCR5 antagonists inhibit HIV attachment by preventing the interaction of HIV-1 gp120 with CCR5 co-receptor used by CCR5 viral strains (R5-tropic) to enter the cell. The molecular antagonists bind in a side pocket region of the CCR5 molecule and cause a conformational change that disrupts the CCR5's molecular recognition of HIV-1 gp120 (37).

1.4 BIOLOGICAL BASIS AND MECHANISM OF DRUG RESISTANCE

HIV-1 has a high mutation rate determined to be $4.1 \pm 1.7 \times 10^{-3}$ per base per cell *in vivo* (38), and this translates to an arbitrary calculation of about 1 - 36 mutations occurring in each RNA

genome target per round of replication. This is due to the error-prone HIV-1 RT enzyme that lacks a 3'- 5' exonucleolytic proofreading (39). The error rate of HIV forms one of the factors for the basis of HIV drug resistance – that is, the virus acquires mutations that enable it to grow in the presence of drugs. Besides, HIV-1 has a high recombination rate when the co-infection of a cell occurs with more than one variant (40). The high recombination rate facilitates an interchange of HIV genomic sequences one or all of which might harbor drug resistance mutations (41). HIV replicates as a pool of diverse viral strains termed as quasispecies that differ slightly in their genetic makeup in an individual (42). The sequence diversity of HIV has led to its systematical classification into two types (HIV-1 and HIV-2); groups (M, N, O, P), subtypes within groups and circulating recombinant forms that may all evolve different mechanisms of acquired resistance mutations and reduce the efficacy of ARV therapy (43).

1.5 HIV-1 RESISTANCE MECHANISMS AGAINST ARV DRUGS

HIV-1 drug resistance mutations can be described as primary (major) or secondary, which encompasses additional descriptive terms, such as minor and accessory or compensatory mutations (40). Most drug resistance mutations occur in the *pol* gene and those that are detected by standard genotypic tests confers resistance to 4 of the ARV drug classes (NRTIs, NNRTIs, PIs, and INSTIs). These 4 ARV drug classes are the main compounds in first and second-line therapy globally. The following section will only describe resistance mechanisms to these drug classes and exclude resistance mechanisms to other classes.

1.5.1 Resistance mechanisms to NRTIs:

The major resistance mutations for NRTIs can be further classified into primer unblocking mutations or discriminatory mutations based on their occurrence near the HIV RT active site (40). Primer unblocking mutations, also referred to as thymidine analog mutations – TAMs, have an effect on ATP-mediated phosphorolytic excision of nucleosides, a process shown to reverse the incorporation of NRTIs (44). TAMs occur near the active site of HIV-1 RT enzyme and are selected by thymidine analogues AZT and D4T. TAMs occur in two pathways - type I or type II. The type I TAMs include M41L, L210W, T215Y show increased high-level of resistance to TDF, ABC and ddI, whereas the type II TAMs, which include D67N, K70R, T215F, K219Q/E are described for intermediate resistance to AZT and D4T(45).

Discriminatory mutations include clinically observed examples, such as K65R and M184V. K65R causes intermediate to high resistance to TDF, ddI, ABC, and d4T and low and intermediate resistance to 3TC and FTC. M184V causes high-level resistance to 3TC and FTC

(46). Discriminatory mutations are so named as they enable a conformational change in the HIV-1 RT active site to 'discriminate' / or identify the NRTI from the cell's native dNTPs (40).

Minor and accessory or compensatory mutations for NRTIs depend on evolutionary selection forces that stem from the host immune system as well as drug pressures. The appearances of these mutations are usually assumed to restore viral fitness, which in turn, is usually affected when the virus acquires major mutations. One example is the A62V mutation that is a non-polymorphic mutation that occurs in HIV-1 A subtype and thought to compensate for viral fitness when it occurs with K65R (46). However, it was shown that A62V has no effect when it occurs with K65R and M184V in HIV-1C (Njenda DT, Master's Thesis, https://scholar.sun.ac.za/handle/10019.1/9866).

1.5.2 Resistance mechanisms to NNRTIs:

The hydrophobic binding pocket for NNRTIs is formed by the amino acid residues L100, K101, K103, V106, T107, V108 V179, Y181, Y188, V189, G190, F227, W229, L234, and Y318 and mutations in the nucleotide positions of one of these sites can potentially cause resistance to NNRTIs (47).

K103N and Y181C are the most common mutations known to cause intermediate to high-level resistance against first (NVP, EFV) and second (ETR, RPV)-generation NNRTIs (46). K103N is an exception as it occurs outside the hydrophobic binding region. The K103N mechanism of action involves a hydrogen bond induced by its presence that prevents entry to the binding pocket region for NNRTIs (48). Minor mutations for NNRTIs usually occur from codon 225 to 318 of HIV-RT enzyme and usually occur with major mutations and have the impact of reducing the susceptibility of NNRTIs (47)

1.5.3 Resistance mechanisms to PIs:

Resistance mutations to PIs occur less frequently in the protease gene in clinical practice (49). The development of key mutations to reduce the binding ability of PIs to the homodimer active site of the protease enzyme occurs near the substrate-binding cleft of the enzyme (50). These mutations have the effect of stretching the cavity of the active site and causing an unstable binding for the inhibitor (51). Common mutations that occur in the protease gene include M46L, I50V, D30N, G48V, V82A, I84V and L90M that have been shown to have clinically relevant HIV-1 drug resistance (52).

1.5.4 Resistance mechanisms to INSTIs:

The catalytic core of the integrase enzyme is stabilized by the geometry of three residues termed the 'DDE' triad (amino acid residues D64, D116, and E152) (53). Resistance mechanisms to INSTIs, develop as a result of conformational changes induced by mutations that occur in the catalytic core, such as N155H and QI48H that increase the binding energy requirements for INSTIs. Other mutations (G118R, Y143C/R) that have been described may impose steric hindrances to INSTIs such as EVG and RAL that have an oxadiazole group within their structure (54). Accessory mutations, such as R263K, may play a role in decreasing the viral DNA binding or may compensate for viral fitness, such as E138A and G140A (55). Other mechanisms of resistance that are currently being explored for INSTIs, such as DTG involve mutations in the LTR region (56). It is hypothesized that mutations in the LTR region could disrupt conventional integration, leading to linear forms of unintegrated viral DNA (possibly containing resistance mutations) that could still maintain a basal expression of the viral mRNA, that in turn will lead to viral production of INSTI resistant viruses (57). Overall, resistant mutations to INSTIs are not well characterized and require more research effort.

1.6 DRUG RESISTANCE CLASSIFICATION

Drug resistance mutations can be classified as pre-treatment drug resistance (also called pre-therapy or baseline drug resistance), and it refers to drug resistance mutations that are detected before a person starts therapy (2). Drug resistance mutations can also be classified as acquired/ therapy-induced resistance (resistance that develops when an individual is receiving ARV therapy over time (58). Pre-treatment drug resistance can be observed as possibility of two scenarios. The first being pre-treatment drug resistance as a result of a transmitted infectious resistant virus from one individual to another - termed as transmitted drug resistance (TDR) (58, 59). TDR can either be horizontal transmission via heterosexual/homosexual intercourse or be vertical via mother to child transmission (MTCT) and may be hard to detect owing to the sensitive limit of standard genotypic assays and the possibility of reversion of drug resistance virus to wild- type forms (60). The second scenario involves pre-treatment drug resistance as a result of undocumented ARV therapy in individuals who present at the clinic as therapy-naïve HIV-1 infected patients. In most cases these individuals are initiated on treatment without genotypic testing, which is usually the standard of care in high-income countries (HMIC), but not routine in LMIC (61).

1.7 DRUG RESISTANCE IN SUB SAHARAN AFRICA

HIV drug resistance remains a challenge for SSA. Figure 3 below presents a map of Sub-Saharan Africa as classified by the world bank with median estimates of pre and therapy failure -associated drug resistance. Pre-treatment and therapy failure-associated/acquired drug resistance mutations reports published from 2010 to date are indicated in tables 3, 4 and 5 below for Northwest, west and Central Africa; Northeast and Eastern Africa and Southern Africa, respectively.

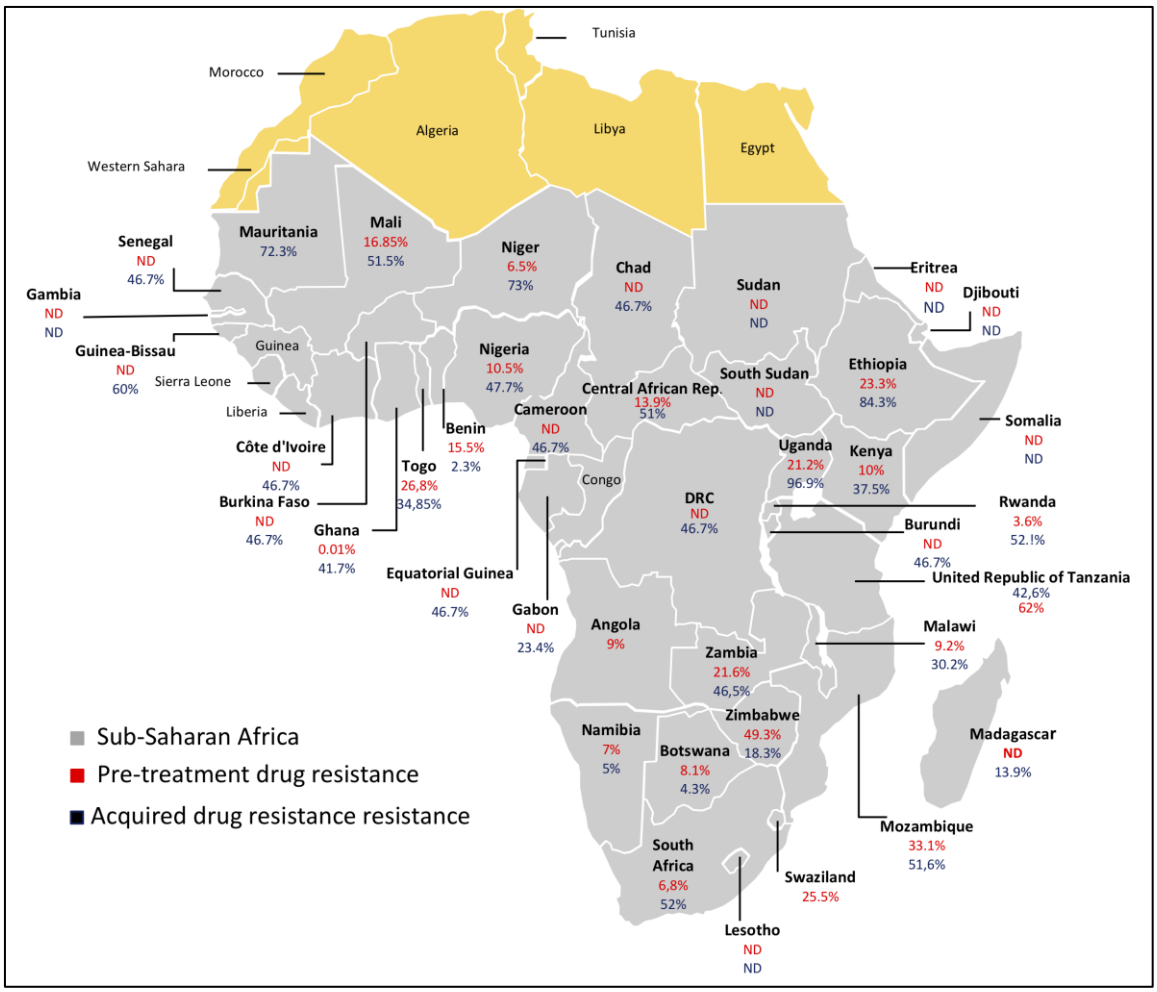

Figure 3. Sub-Saharan Africa median estimates of pre-treatment drug resistance (in red) and therapy failure-associated (acquired drug resistance[ADR]) (in blue). Drug resistance range values are included in table 2-4 with references from the literature. ND = no data available

1.7.1 Northwest, West and Central Africa

The expansion of antiretroviral programs has been a challenge in Sub-Saharan Africa countries in the northwest, west, and central region of Africa due to the high cost for acquisition of drugs, lack of virological monitoring and inadequate laboratory infrastructure (62). The presence of multiple HIV-1 subtypes adds to the problem of performing timeous screening of infected individuals as well as achieving sustained viral suppression due to the different observed efficacies that different ARVs have on non-B subtypes (63). A recent report that included 3736 sequences from central African countries (Burundi, Cameroon, Chad, Democratic Republic of Congo, Equatorial Guinea) and northwest and western African countries (Benin, Burkina Faso, Cote d'Ivoire, Senegal and Togo) had no indication of pre-treatment drug resistance, but indicated the frequency of acquired major resistance mutations to NNRTIs and NRTIs to range from 5.5 – 88.9% (64). The previously mentioned report had good concordance with data from the 2LADY- ANRS12169/EDCTP clinical trial. The trial had participant patients from Burkina Faso, Cameroon and Senegal and reported a 98.7% prevalence of at least one drug-resistant mutation to first-line therapy (AZT/d4T+3TC + EFV/NVP) and a therapy failure rate of about 77%. Therapy failure due to drug resistance in the 2LADY- ANRS12169/EDCTP clinical trial was about 50 % of the therapy failure cases (65).

Table 2. PDR and ADR rates for northwest, west and central Africa

Country	Pre-treatment drug resistance	Acquired drug resistance
Benin	15.5% (66)	2.3% (67)
Cape Verde	3.4 -12% (68)	10-47.8% (HIV-1);17.6% (HIV-2)(68) (69)
Central African Republic	13.9%(70)	26-76% (71, 72)
Gabon	Data unavailable	Calculated to 23.4% (73)
Ghana	0.01% (74)	41.7% (75)
Equatorial Guinea	3.2 -16.1% (76, 77)	12.7% (78)
Mali	7.9 – 25.8% (79, 80)	11-92% (81-83)
Mauritania	Data unavailable	72.3% (84)
Niger	6.5% (75)	73% (85)
Nigeria	<5 – 15.9% (86, 87)	1.6-93.8% (88-92)
Sierra Leone	Data unavailable	Data unavailable
Togo	26.8% (93)	18.1 -51.6% (94)
Gambia	Data Unavailable	Data unavailable
Guinea- Bissau	Data Unavailable	60%(95)
Liberia	5.9% (96)	63-71% (97)

1.7.2 Northeast and Eastern Africa

Table 3. PDR and ADR rates for the northeast and eastern Africa

Country	Pre-treatment drug resistance	Acquired drug resistance
Kenya	1.1 -10.93% (98-102)	7 -68% (103-105)
Ethiopia	6.5 – 40% (106, 107)	81.8 – 86.7% (108, 109)
Eritrea	Data unavailable	Data unavailable
Somalia	Data unavailable	Data unavailable
Uganda	1.4 – 41 % (110-117)	93.8 -100% (118-120)
Sudan	Data unavailable	Data unavailable
South Sudan	Data unavailable	Data unavailable
Rwanda	3.5 – 3.6 % (121)	9.1 -95 % (122-124)

1.7.3 Southern Africa

Table 4. PDR and ADR rates for southern Africa

Country	Pre-treatment drug resistance	Therapy failure associated resistance
South Africa	4.6- 9% (125-127)	52% (128)
Lesotho	Data unavailable	Data unavailable
Swaziland	9.1-41.9% (111)	Data unavailable
Mozambique	5.4-60.7% (111, 129)	10.1-93% (130-132)
Zimbabwe	23.9-74.7% (111)	6-30.6% (133, 134)
Botswana	3.5 -5% (135, 136)	6.6 -9.6% (136)
Namibia	7% (137)	5% (137)
Zambia	3-40.2% (138-140)	5-88% (141)
Angola	1.6-16.3% (142, 143)	Data unavailable
Malawi	<5- 13.3% (144, 145)	20.3-40% (129, 146, 147)
Tanzania	2.2-82.9% (148-151)	34-90% (152-154)
Madagascar	Data unavailable	13.9% (155)
Mauritius	Data unavailable	Data unavailable
Comoros	Data unavailable	Data unavailable

1.7.4 Additional challenge for SSA to the success of ART therapy

The projected success of ARV therapy is envisioned under the UNAIDS 90-90-90 goals (156). However, there are a few additional problems that challenge the overall success of ART for Sub-Saharan Africa, and these include drug-drug interactions, adherence, limited development of research infrastructure and health care delivery systems and increasing prevalence of diverse HIV-1 subtypes. These are briefly mentioned below:

1.7.4.1 Drug-Drug Interactions (DDI)

The treatment of opportunistic infections from bacteria and other viruses in HIV-1 infected patients compromises the effectiveness of ARV therapy via drug-drug interactions and this may lead to the emergence of drug resistance (157, 158).This is particularly common in the Sub-Saharan region, where there are high prevalence rates of tuberculosis (TB), malaria, hepatitis and fungal infections (159). An example is the simultaneous administration of TB drugs (rifampicin –RMP, isoniazid- INH, pyrazinamide –PZA, ofloxacin, levofloxacin and moxifloxacin) with ARVs (160).

Studies have shown that rifampicin is a potent inducer of CYP2B6 and CYP3A4 liver enzymes and its co-administration with ARVs, such as NVP and ATV, leads to a reduction in the plasma levels of the ARV drug (161, 162)

1.7.4.2 Adherence

Lack of drug stocks, ARV-related toxicities leading to adverse reactions and poverty characterize some of the problems that lead to non-adherence in HIV-1 infected patients in Sub-Saharan Africa (163). Access to ARVs from remote locations also plays a factor and studies have shown a relationship between the distances to near-by healthcare facilities from an HIV infected patient's residence relates to their probability to a loss- to- follow-up (LTFU) impacting their ability to access ARVs (164). None and sub-optimal adherence lead to the evolution of drug resistance and faster rates of progression towards virological failure (165). Other factors include influences of traditional medicine, faith-based healing, alcohol abuse and poverty (166) (which often leads to depression and food insecurity) are described in a meta-analysis study (167) as 'bottlenecks' for achieving high adherence in HIV-1 infected patients.

1.7.4.3 Limited infrastructure development

The development of viral load monitoring and genotypic resistance testing facilities is crucial for the success of early initiation of ARV therapy (168). However, most Sub-Saharan African countries are lagging in this endeavor. Given that there is an increasing level of HIV-1 diverse types that require efficient and timeous detection of new HIV infections, the success and implementation of national ARV programs will largely rely on coordinated efforts of government support and expert contribution from healthcare providers (169).

1.8 HIV-1 PREVALENCE AND DRUG RESISTANCE IN SOUTH AFRICA

South Africa has the largest antiretroviral program in the world. About 4.4 million individuals are receiving treatment and only 47% have achieved viral suppression below the detection limit as determined by clinical assays (170). HIV-1 subtype C is the dominating strain, but more and more circulating recombinant forms are emerging (171). HIV prevalence rate in South Africa remains one of the highest in the world and is approximately 19% for adults aged 15-49 nationally, with some remarkable differences between individual provinces in South Africa (Figure 4) (172).

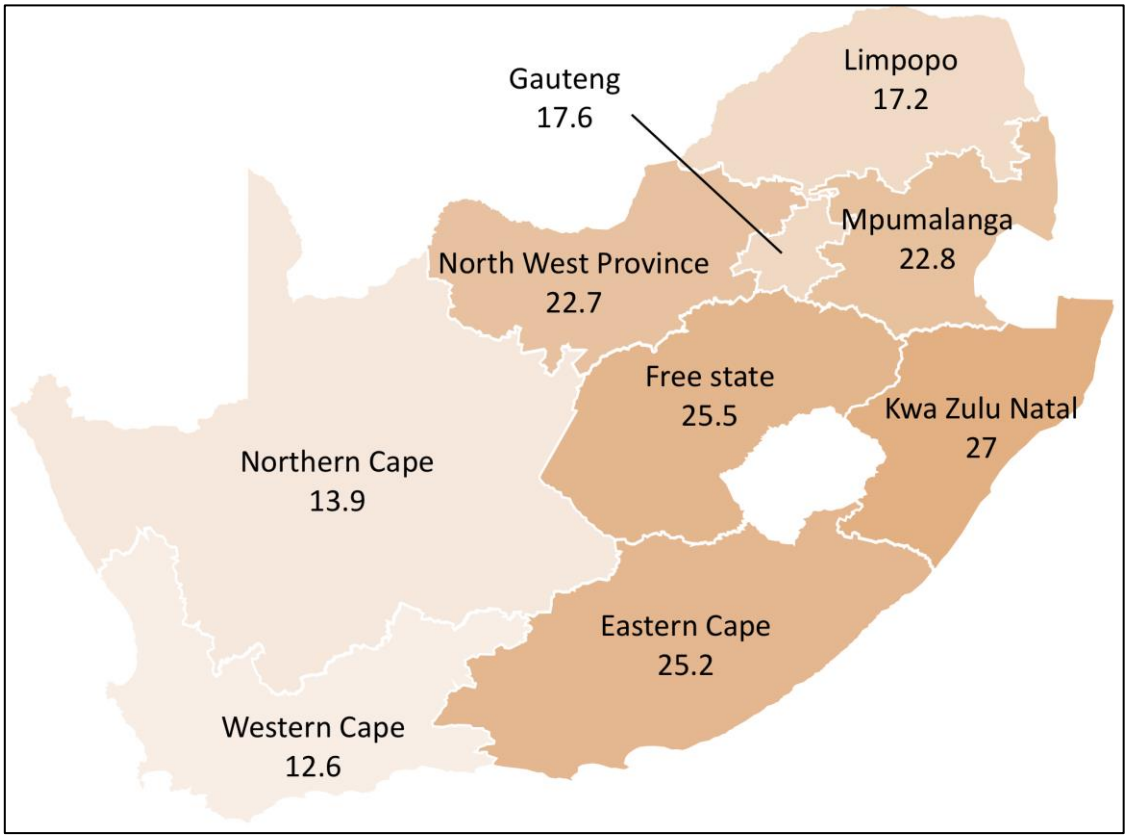

Figure 4. HIV-1 prevalence by the province in South Africa (172)

Pre-treatment drug resistance (PDR) has increased since the country's national ARV rollout program in 2004 and this increase, is specifically evident in HIV pregnant women receiving NNRTIs or a combination of NNRTIs and NRTIs (173).

The hardest-hit provinces in South Africa when it comes to PDR are Gauteng (162) and Eastern Cape (174), followed by Mpumalanga (175) and KwaZulu Natal (173) and lastly by Limpopo (176), Western Cape (127) and the Free State (177) (Figure 5).

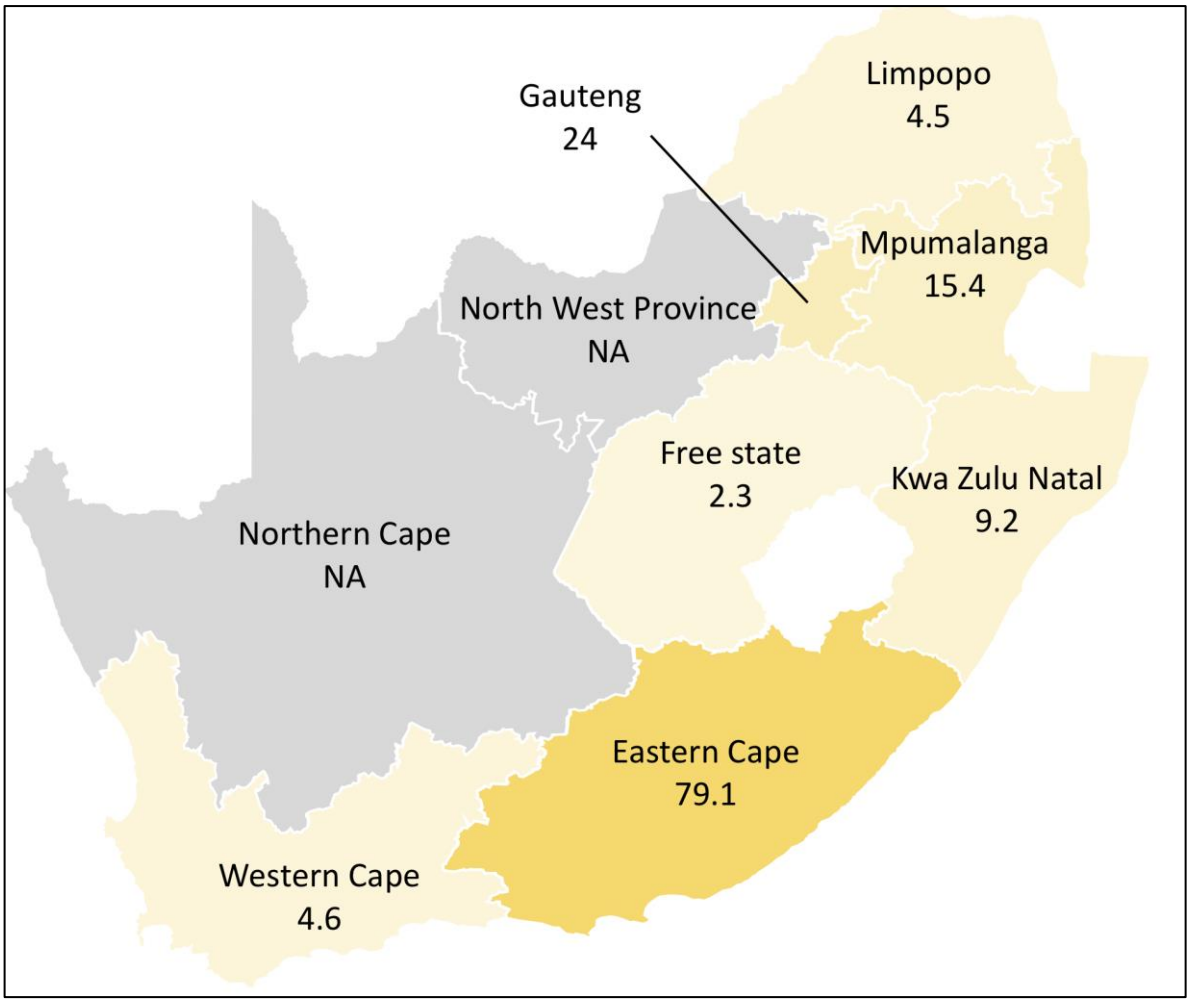

Figure 5. HIV-1 PDR percentage rates by province in South Africa. NA = no data found/available

Projections for South Africa for HIV-1 infected patients requiring second-line protease inhibitor (PI)-based boosted treatment will increase to over 900000 by the year 2030 from the current estimate of about 128000 individuals that are now on PI-based therapy (178). The major challenge for managing HIV-1 patients on PI-based treatment is to make determinations for therapy failure, considering that major protease-resistant mutations are rarely present at treatment failure (179).

1.10 HIV-1 PREVALENCE AND DRUG RESISTANCE IN SWEDEN

The HIV-1 burden in Sweden has reduced drastically since the introduction of combination therapy, and Sweden is the first country to reach the UNAIDS/WHO 90-90-90 goals (180). With a prevalence of less than 0.2%, Sweden is mainly dominated by HIV-1 Subtype B, though there is evidence that circulating recombinant forms are emerging and are being driven by specific transmission clusters (181). The central transmission cluster is with Men who have sex with other men (MSM) that are estimated to be around 100000 in Sweden (182).

The prevalence of HIV-1 drug resistance in Sweden remains low, but shows a significant increasing trend from less than 1.1% (183) to about 7.1 % with the majority of resistant carriers believed to be immigrants coming from SSA and MSM clusters (184). Most of the HIV-1 drug resistance is reported for patients who are using NRTI and NNRTI -based regimens and using ultra-sensitive genotypic resistance methods (107). This has led to recommendations for HIV-1 patients who have immigrated to Sweden, particularly from SSA to be initiated on PI-based regimen (184). However, the risk of virologic failure has been shown to be high in HIV non-B subtypes (185) and recommendations to initiate patients on PI regimens will still require close monitoring and frequent genotypic resistance testing in suspected cases of failure.

Overall, HIV-1 research in Sweden still has a significant role and is required to understand the dynamics of transmission of non-B HIV-1 subtypes as well as mechanisms of drug resistance that could ultimately change the HIV-1 Swedish epidemic in the foreseeable future.

The next chapter will focus on the specific role of gag and protease in affecting the development of HIV-1 resistance to ARV drugs.

2 ROLE OF GAG AND PROTEASE IN DRUG RESISTANCE

2.1 HIV-1 GROUP ANTIGEN PROTEIN (GAG)

HIV-1 gag is a structural gene whose precursor (designated as gag $Pr55^{Gag}$) has a molecular weight of 55kDa and is cleaved into mature proteins - Matrix (MA), Capsid (CA), nucleocapsid proteins (NC), p6 and spacer proteins p1 and p7 by the viral protease encoded by HIV-1 pol gene (186). The Gag precursor is thought to have assembly and membrane targeting functions, whereas the mature proteins are involved in uncoating and disassembly (186). There are approximately 2000 copies of Gag in a single HIV-1 virus particle (187) that comprises about 50% of the viral mass (188).

HIV-1 resistance mechanisms to PIs have been proposed to include the development of mutations in the gag gene at protease cleavage sites (189). In total, there are 12 protease cleavage sites within the HIV genome, and 5 of them are in the *gag* gene (190, 191). Gag mutations at cleavage sites are hypothesized to cause an interaction with the active site of protease reducing the binding affinity of PIs and thereby allowing the enzyme to resume its proteolytic function (192-194). Gag cleavage site mutations reported include V128I (MA/CA); S373P,I376V (p2/NC); A431V and K436R (NC/p1) and P453A(p1/p6)(195) and are thought to increase PI resistance when occurring with primary drug resistance mutations in protease (189).

Another mechanism involves the development of *gag* mutations that act as secondary or compensatory mutations and restore viral fitness (189). One example of compensatory *gag* mutations, is a study involving the PI darunavir (DRV), the observation of gag mutations H219Q and I223V appeared to compensate for viral fitness in the presence of primary protease mutations (196). Other studies had shown that there is a minimal association of *gag* cleavage site mutations with primary resistance mutations and that *gag* non-cleavage mutations play a role (197). The most common *gag* non-cleavage site mutations characterized (mainly in the MA and CA regions) include T242N/S(198); T427D/N, E46V/K, Q369L(199); R76K, Y79F, and T81A(200). In a recent longitudinal study involving four patients from Spain who had been treated with PIs for nine years, the co-evolution of MA and CA non-cleavage site mutations was strongly associated with protease mutations (201) implying the role these mutations may have in impacting PI-based therapy.

In addition to the MA and CA regions, the C-terminal of *gag* involving the p6 has also been implicated in conferring resistance to ARVs in a similar mechanism that restores viral fitness.

One example, is a study that examined the association of *gag* p6 mutations P5L/T and K27Q/N in enhancing p6 function in packaging pol molecules and reducing the potency of NRTI-based therapy against HIV CRF02_AG and subtype G (202). Further evidence of the role of *gag* p6 in resistance and restoration of viral fitness is provided by studies that found amino acid motif duplications and insertions in the p6 gene. The most common of these motifs in p6 that provide an escape mechanism for increased incorporation of RT molecules include PTAPP duplications (203, 204). More recent studies have described a PYxE insertion in *gag* p6 of HIV-1 subtype C that enhances viral replication and is associated with protease failure (205, 206).

Overall, much remains unknown about cleavage, non-cleavage and c-terminal mutations in gag and the role they play. However, there is a strong link between *gag* and protease mutations, and this is particularly important as PIs are still used in the clinical setting and hence understanding patterns and mechanisms of resistance to PIs is crucial to ensure the management of HIV-1 patients.

2.2 HIV-1 PROTEASE

HIV-1 Protease is a homodimeric aspartic enzyme with a molecular weight of 21.6kDa that catalyzes the cleavage of gag and Gag-Pol polyproteins (207). The catalytic site of the HIV-1 protease interacts with the protease inhibitor (PI). Mutations in the active site that lead to protease resistance against PIs are designated to be primary mutations, whereas those outside are termed to be secondary mutations (208). Primary mutations decrease the proteolytic function of protease reducing the replication capacity of the virus. Primary protease mutations are affected by amino acid residues 25-32, 47-53 and 80-84 of protease (209). Secondary or minor mutations can be pre-existing as naturally occurring polymorphisms (NOPs) or can develop after primary mutations have appeared in the presence of PI treatment (208). Most studies on the development of PI resistance have observed that secondary mutations have a compensatory effect on the fitness of protease instead of conferring resistance directly and occur with a frequency range of 40-45% for the entire Protease amino acid sequence (209). One example is a mutagenesis study the reported the impact of secondary mutations M36I and A71V when co-occurring with the primary mutation D30N to have more than 10-fold increase in resistance against the PI drugs nelfinavir and ritonavir (210).

Finally, it's unclear why specific mutations impact Protease extensively in some HIV-1 subtypes and not in others. For instance, the mutation L90M in combination with D30N and N88D had a more significant impact on increasing resistance to PI drugs in HIV-1C compared to HIV-1B (211). One explanation for this is that the plasticity of HIV-1 Protease allows for

various permutations of mutations in both the primary and secondary sites to have an impact on the conformation of the active site and its hydrophobic interactions with the inhibitor (209). How this interaction differs amongst HIV -1 subtypes is a question yet to be fully answered.

The next chapter will look at some newer drugs that have the potential for use as long-acting drugs and alternatives to PIs for treatment in HIV-1 non-B subtypes.

3 NEWER ANTIRETROVIRAL DRUGS

3.1 EFDA

EFdA (4'-Ethynyl-2'-Fluoro-2' deoxyadenosine) also known as MK-8591/Islatravir is a result of a modification of one of 4'-ethynyl compounds described initially to act as NRTIs and shown to have potent activity against HIV-1, drug-resistant strains of HIV-1 (212) and more recently HIV-2 (213). Structurally, EFdA is distinct from other classical NRTIs in that it retains a 3-OH group in its ribose sugar and has an ethynyl and fluoro group (214). Chemical synthesis of EFdA has been improved over time from a yield of 2.5% to 37% (215). Currently, using less costly starting material and fewer purification steps, EFdA synthesis has an overall yield of 90% (216), allowing mass production of the drug form clinical applications.

Recent studies have confirmed that EFdA has multiple modes of HIV-1 inhibition and has been termed to be a translocation-defective reverse transcriptase inhibitor (TDRTI) based on one of its mode of inhibition. The first mode of inhibition involves the triphosphate form of EFdA (EFdA-TP) acting as a direct chain terminator, whereas the second mode involves EFdA-TP acting as a delayed chain terminator in a template sequence-dependent manner (217). The third mechanism of inhibition involves its monophosphate form (EFdA-MP) being incorporated in the primer used by HIV RT for the extension. The insertion of the monophosphate form in the primer leads to a mismatch that makes it hard for HIV RT to copy the template and to excise the monophosphate form of EFdA (217). As an attractive candidate ARV drug for clinical application, EFdA has been shown to have low toxicity and high resistance to deamination by adenosine deaminase (increasing its intracellular half-life(~17.5 to >72h)) – a characteristic that enhances its antiviral activity significantly more than other NRTIs (214). *In vitro* studies looking at the intestinal absorption and permeability of EFdA have shown that the drug mainly uses the paracellular route (characterized by compact spaces between cells and primarily used by small molecules for passive diffusion) (218). *In vivo*, EFdA has been shown to have efficacy in the gastrointestinal and female reproductive tracts of humanized bone marrow, liver and thymus mice (219). Additionally, it was also shown in rhesus macaques that EFdA had strong antiviral activity against simian immunodeficiency virus (SIV) for extended periods at sub-nanomolar concentrations (220).

EFdA has already entered human clinical trials. Phase I clinical trial evaluating the dosing and safety profiles of EFdA concluded that EFdA did not result in any adverse effects and could be tolerated even when administered up to 30mg per day (221). Phase II studies are being

conducted as of the time of this writing. It is worth noting that the systematical evaluation of EFdA in comparison to NRTIs and NNRTIs has not been fully explored.

3.2 BICTEGRAVIR

WHO has recently advocated for the use of INSTIs as part of first-line therapy in low- and middle-income countries (2). It is worth noting that INSTI-based-regimens have been shown to have the same efficacy with PI-based regimens (222).

Bictegravir (BIC), a new INSTI drug was developed as a result of limitations observed with first-generation INSTI drugs Raltegravir (RAL) and Elvitegravir (EVG). These limitations include a low genetic barrier to resistance for all first-generation INSTIs, the requirement for twice-daily dosing specifically for RAL and the need for pharmacokinetic enhancement for EVG (223, 224). Problems with the second generation INSTI drug – Dolutegravir (DTG), that included drug-drug interactions, increase in patient serum creatine levels (223) and neural-tube defects in neonates (225) also encouraged the development of BIC. Structurally BIC has different substituted groups in its benzyl tail and a cross-linked bicyclic ring that reduces its chances to activate the pregnane X receptor (PXR) and lowers the risk of drug-drug interactions (224).

The mechanism of action of BIC involves blocking the active site of HIV-1 Integrase, consequently impairing its stand-transfer ability, but BIC acts as a weak inhibitor for the 3' processing activity (224). *In vitro* studies have reported BIC to have a longer half-life of dissociation from Integrase/DNA complexes compared to RAL, EVG and DTG (226). In clinical studies, the safety profile for BIC has been evaluated in a 10-day phase Ib study and it was shown that BIC was tolerated when doses of up to 100mg per day were administered and no INSTI associated mutations were observed (227). However, in a 24-week phase II study which compared the fixed-dose combination of BIC with FTC and TAF against DTG, FTC and TAF, an interesting observation on the adverse effects BIC could potentially have in the long term was observed. Though perhaps not statistically relevant one patient in the 24-week clinical trial taking BIC, FTC and TAF developed urticaria (228). However, HIV-1 prevalence and transmission resistance studies reported no clinical resistance development for BIC and have advocated for no baseline resistance testing for patients initiating BIC regimen as first-line therapy (229). Phase III clinical study of BIC containing regimen compared to DTG containing regimen indicated that BIC was not inferior to DTG and that it was equally tolerated with no adverse effects significantly related to its use (230). To date, HIV-1 subtype-specific differences for BIC have not been evaluated extensively.

3.3 CABOTEGRAVIR

Cabotegravir (CAB) (also known as GSK1265744) is an INSTI drug with the same mechanism of action as DTG and BIC. CAB is mainly degraded by uridine diphosphate glucuronosyltransferase (UGT) 1A1 and has an approximate half-life of 40 days (231).
The crystalline form of CAB has been used to develop the long-acting form that is administered subcutaneously as nanosuspension particles of the drug (231). An *in vitro* study assessed the potency of CAB against five different subtypes of HIV-1 derived from treatment naïve patients and reported a mean EC_{50} of 0.91nM (232). To add on, the previously mentioned study indicated that CAB had high EC_{50} and EC_{90} values to RAL-resistant viruses that contained G140S and Q148H integrase mutations. In contrast, it was shown in another study (233) that CAB had a lower EC_{50} compared RAL and EVG for G140S and Q148H-carrying viruses. On the other hand, *an in vivo* animal study showed that CAB long-acting administered prophylactically, protected macaques from SIV infection (234). Although a different study emphasized caution and pre-therapy testing before CAB is given as prophylaxis as in the study's SIV animal model, some macaques developed resistance to CAB and could not protect against viral infection (235).

Phase I clinical trials demonstrated that up to 30 mg of CAB could be tolerated with no adverse effects (236) and a supratherapeutic dose of up to 150mg every 12 hours given to healthy individuals, did not affect the cardiovascular system (237). Other phase 1 studies, have shown CAB had no adverse effect on the liver (238) and kidney function (239). A Phase II study reporting tolerability and pharmacokinetics of injectable CAB had a significant number of patients discontinuing treatment due to injection site reactions (ISR) (240). In the LATTE-2 phase II study, CAB was paired with RPV and proved to be as effective as EFV combined with two NRTIs (241, 242).

Regardless of support data, from clinical studies on the safety, tolerability, favorable pharmacokinetics, and antiviral efficacy of CAB, more research is required to understand the resistance mutations that can develop as a result of continued use of CAB. This will be crucial in situations where CAB long-acting-based treatment is given with other drugs treating co-infections as it is predicted that it might cause drug-drug interactions (243).

I will now present my findings for my thesis in the next chapter.

4 PRESENT FINDINGS

4.1 THESIS AIMS AND OBJECTIVES

Antiretroviral drug development and most of the clinical trials are mainly restricted to the HIV-1 subtype B (HIV-1B) dominating high- income countries. The genetic and replicative capacity differences among subtypes can lead to the emergence of subtype-specific resistance mutations, which frequently also alter viral fitness (244-246). Substantial evidence supports the impact of genetic diversity on ART responses and drug-resistance pathways (247-249).

I hypothesized that HIV-1 subtype-specific differences can impact the susceptibility towards antiretroviral drugs that can affect the treatment efficacy and alter the drug resistant mutations pathways in non-B HIV-1 subtypes significantly.

The overall aim of my thesis is to investigate the potency and drug resistance susceptibility profile of clinically relevant and new ARV drugs that can potentially be used as second-line therapy in non-B dominating countries. The specific aims include the following:

1. To understand the role of PYxE-mutations that was identified by Neogi et al. (206) in viral-fitness and drug susceptibility. The PYxE (where x represents a lysine [K], a glutamine [Q] or an arginine [R]), occurs naturally in HIV-1C viruses from Eastern Africa (Ethiopia and Eritrea) but acquired more in HIV-1C infections from India and South Africa when patients failed on treatment mainly with PI-based regimen (**Paper I**).
2. To investigate the virological and biochemical inhibitory potentials of a new reverse transcriptase inhibitor 4'-Ethnyl-2'-fluoro-2'-deoxyadenosine (EFdA) against broad-spectrum HIV-1 chimeric viruses of different subtypes (**Paper II**).
3. To understand the ex vivo antiretroviral potency of the newer INSTIs Cabotegravir and Bictegravir in non-B HIV-1 subtypes (**Paper III**).

The studies aim to understand the potential subtype-specific differences in viral fitness and drug susceptibility against the drugs that target different stages of viral replication that includes, protease-mediated cleavage and maturation (**Paper I),** reverse transcription (**Paper II**) and integration (**Paper III**) (**Figure 6**).

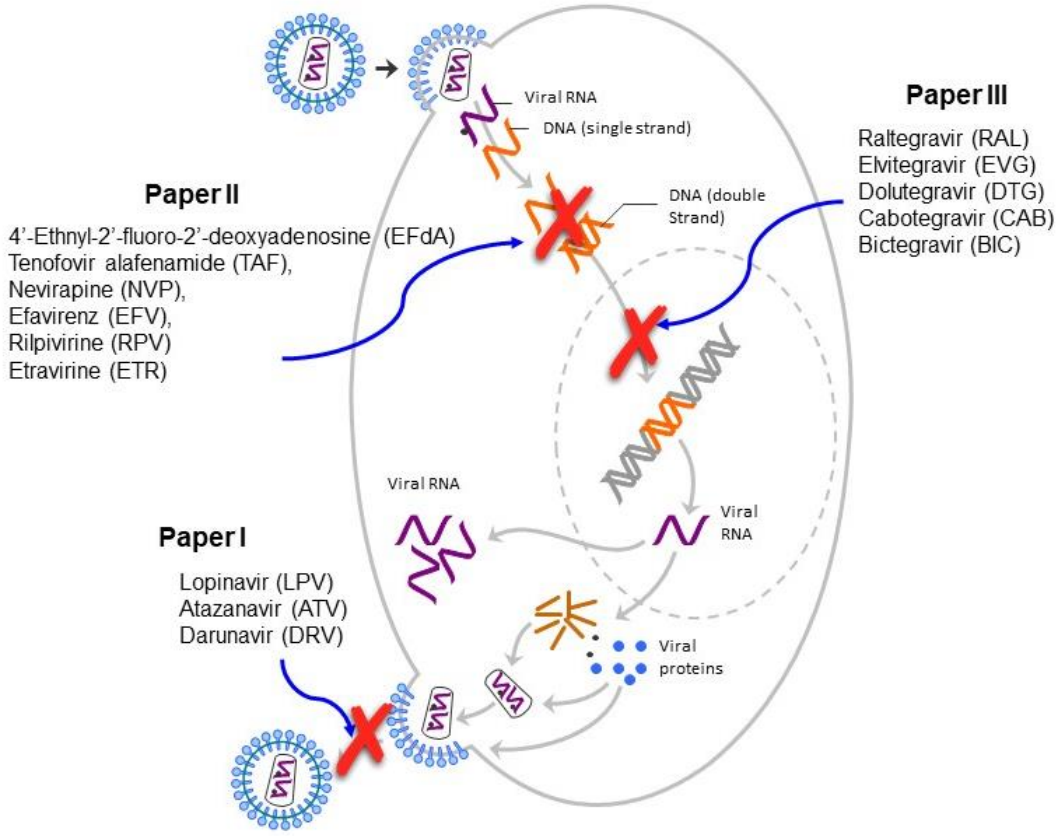

Figure 6: Thesis overview

4.2 MATERIALS AND METHODS (PAPERS I, II, III)

4.2.1 Ethical considerations

For **paper I, II and III** ethical clearance was granted by the Stockholm Regional Ethics Committee. The ethical permits were designated under the following numbers: 2005/1167-31/3 and 2014/928-31/2 for Paper-I; 2014/928-31/2 and 2013/1944-31/4 for paper II; and 2006/1367-31/4 and 2014/928-31/2 for Paper III.

4.2.2 Statistical analysis

In **paper I, II,** and **III**, non- parametric statistical analysis was performed for drug sensitivity and viral growth kinetics assays using GraphPad Prism v6 and 8 (GraphPad Inc. La Jolla, USA).

4.2.3 RNA extraction and synthesis of recombinant viruses

Recombinant viruses (n=24) that were used in all papers**,** were derived from patient samples obtained from the Swedish InfCare cohort that includes patients who have migrated from other Sub-Saharan African countries (inclusive of South Africa) and Asia. Briefly, the recombinant viruses were derived by amplifying the gag-pol region using a previously described PCR protocol (251) and cloned into the pNL4.3 infectious vector backbone using BssHII and SalI restriction sites (**Figure 7**).

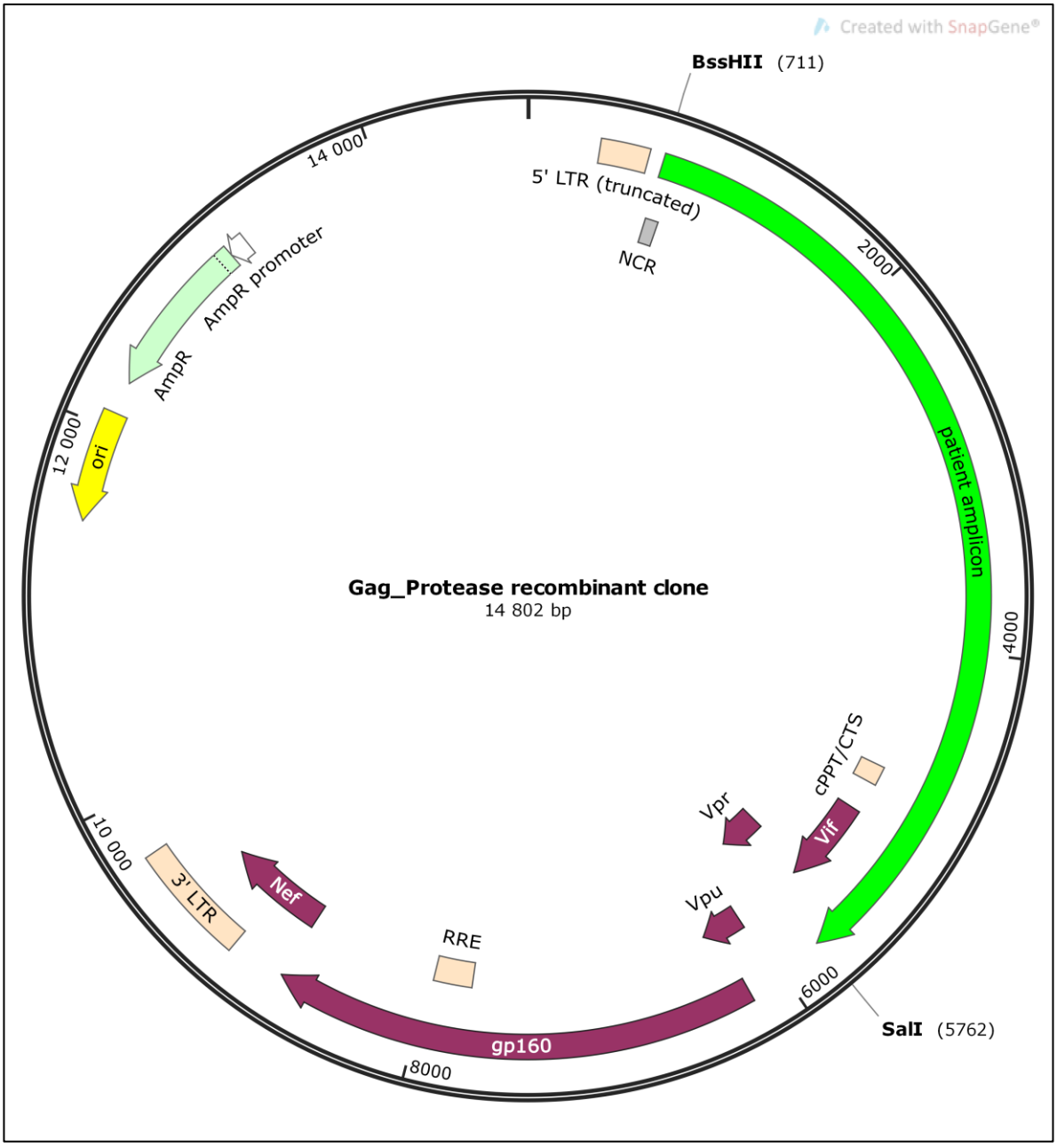

Figure 7. Schematic of recombinant plasmids used in all papers presented in the thesis. Image generated using Snapgene software (SnapGene software from GSL Biotech; available at https://www.snapgene.com/)

4.2.4 Sanger sequencing and analysis

Patient amplicons spanning the gag-pol of recombinant viruses from the Swedish cohort were sequenced using published primers as previously described (251). Sequence fragments were assembled using BioEdit sequence alignment editor (252), and phylogenetic analysis was done using Mega X (253).

4.2.5 Cell culture and reagents

For **papers, I, II and III,** recombinant plasmids were transfected in HEK293T cells to generate viral stocks. HEK293T cells were propagated in 10% fecal calf serum Dulbecco's modified Eagle medium (DMEM, Sigma, USA) supplemented media. TZM-bl reporter cells were also propagated and maintained with the cell culture media mentioned above. The TZM-bl reporter cell line is derived from HeLa cells and stably expresses high levels of CD4, CXCR4 and CCR5. In addition, the TZM-bl cell line has integrated copies of the luciferase gene under the control of the HIV-1 promoter gene that enables the indirect quantification of HIV-1 infection when the HIV-1 promoter is activated. To add on, a panel of NRTI and NNRTI-resistant viruses were obtained from the National Institutes of Health (AIDS reagent program, NIH, USA) and used in viral infectivity and drug sensitivity assays.

4.2.6 Viral Infectivity and drug sensitivity assay (DSA)

The following ARV drugs were purchased from Selleckchem, USA: for drugs used in the **paper I:** ATV, DRV, and LPV; NVP, EFV, ETR, RPV and TAF. were also purchased from Selleckchem, USA and used in **paper II.** EFdA was provided by Professor Stefanos G. Sarafianos (University of Emory). For **paper III,** BIC and CAB were provided by ViiV Healthcare/GSK (Research) Triangle Park, North Carolina, USA) and Gilead Sciences (Foster City, California, USA), respectively. RAL, EVG, and DTG were purchased from Selleckchem, USA. The DSA was performed by determining the extent to which the antiretroviral drugs inhibited the replication of the reference virus (pNL4.3) and *gag-pol* derived recombinant viruses. The DSA was performed by having three technical replicates (triplicate) for each virus added on a 96-well cell culture plate. Each triplicate was added at a specified concentration within the dynamic range of the drug for the assay before the assay plate was incubated at 37˚C for 48 hours. End-point detection of the plate was done using Bright glo™ Luciferase assay kit (Promega, USA). The output at endpoint detection is relative light units (RLU) and is proportional to infectious virions produced in the DSA. The drug concentrations required for inhibiting virus replication by 50% (EC_{50}) is then calculated using the RLUs by a dose-response curve plotted using non-linear regression analysis in GraphPad Prism, version 6.07 (GraphPad

Software, USA). The DSA described in all papers was performed at least three times and the output for the drug EC_{50} results was used to compute the fold change value (FCV) for each virus relative to pNL4-3. PI DSA requires two rounds in contrast to RTI and INSTI drugs as shown in **Figure 8.**

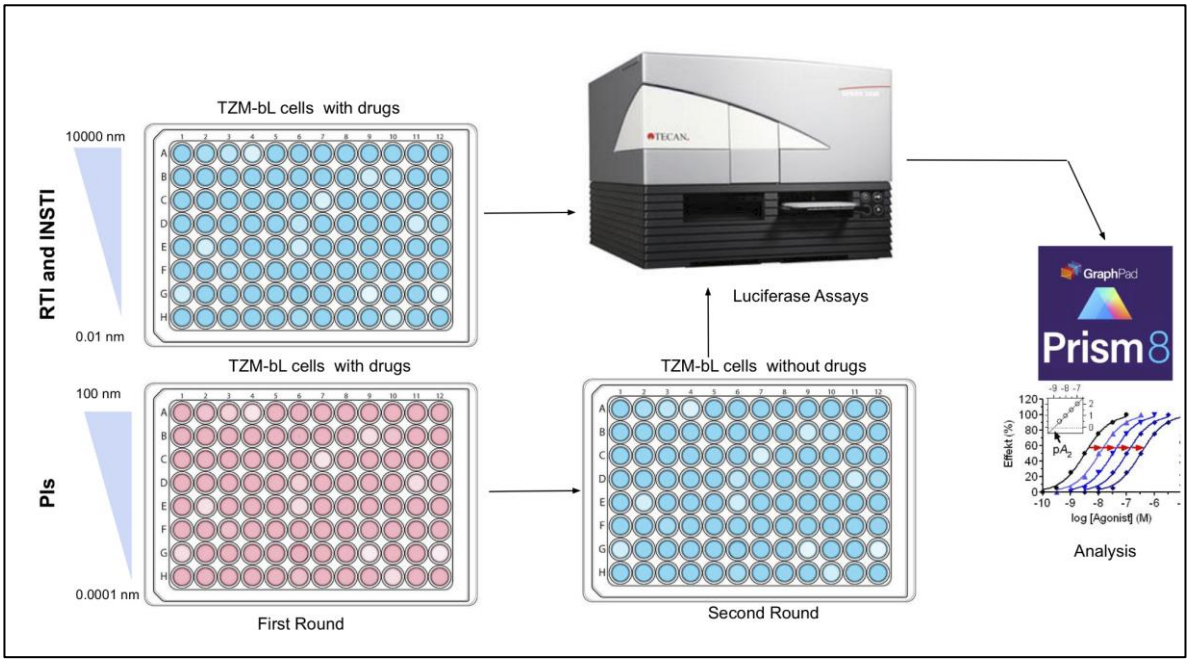

Figure 8. Schematic representation of drug sensitivity assay (DSA).

4.2.7 Viral growth kinetics assays (VGK)

Viral growth kinetic assay performed in **paper I** was done using MT4-cells and the amount of p24 from supernatants collected on days 0, 3, 5- and 7-days post-infection was used as the end-point to calculate viral fitness relative to control reference virus, pNL4-3. Viral fitness was calculated using the formula described by Koval et al. (254) and graphical representation of the result was made using GraphPad Prism v6 (GraphPad Inc., La Jolla, CA, USA).

5 RESULTS (PAPERS I, II, III)

5.1 PAPER I

Article title

HIV-1 subtype C with PYxE insertion has enhanced binding of Gag-p6 to host cell protein ALIX and increased replication fitness

Publication: Journal of Virology

Publisher: American Society for Microbiology (ASM)

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Authors list

van Domselaar Robert*, **Njenda *T. Duncan****, Rao Rohit, Sönnerborg Ander, Singh Kamalendra, Neogi Ujjwal,

***Equal contribution**

Author Contribution and declaration

In the enclosed manuscript; Mr. van Domselaar. and I, are joint-first authors, with equal contributions to the work performed. I performed the virological experiments, including viral growth kinetics and drug sensitivity assays. Mr. Van Domselaar performed the Immunofluorescence, colocalization and co-immunoprecipitation assays in the article. Mr. Rao and Mr. Singh made contributions to the generation of results by *in silico analysis* and microscale thermophoresis assay. Mr. Neogi and Mr. Sönnerborg made contributions to the clinical data used in the article. Mr. van Domselaar and I contributed to the first draft and was responsible for subsequent manuscript updates received from the co-authors

Background: The gag-p6 late domain region of gag interacts with the host cell protein ALG-2-interacting protein X (ALIX) to facilitate active transport of gag to the internal surface of the cell membrane during HIV viral assembly and budding (17). It was previously shown that HIV-1C has a unique PYxE (x being Arginine (R) or lysine (K) or glutamine (Q) amino acid residue) motif insertion in its gag-p6 gene where the YPx_nL motif has been naturally deleted in HIV-1C viruses and that the PYxE motif is associated with a reduction in PI-based therapeutic response in some HIV-1C infected patients (206). Hence in **paper I**, the distribution of PYxE in three HIV-1C cohorts was investigated and in addition the occurrence

of the motif in the gag-p6 region in HIV-1C recombinant viruses was investigated to assess its impact on viral fitness

Main Findings: the prevalence of PYKE in patient viral sequences from three HIV-1 cohorts (Swedish, German and Ethiopian) was higher than PYQE (~2 fold). In addition, clinical data from the Swedish cohort looking into patients specifically infected with HIV-1C revealed that the viral load and the $CD4^+$ T-cell count were higher and lower respectively, in patients carrying PYxE viruses compared to the wild-type (WT) HIV-1C. *In silico* simulation and co-localization experiments performed aimed to previse and show the interaction of ALIX and gag (PYxE). The interaction with ALIX was especially enhanced with the presence of the PYxE motif compared to HIV-1C WT. Furthermore, it was shown that there is a high binding affinity between ALIX and gag (PYxE), and this affinity correlated well with the observed increased replication capacity of recombinant HIV-1C containing the PYxE motif when compared with non-C viruses. More specifically, viral growth differences were noted for HIV-1C viruses with PYKE, PYQE, no PYxE (i.e. WT) and when compared with the reference HIV-1B WT. Chimeric viruses with PTAPP duplication (PTAPPd) were also included in the assessment as this had been previously shown to impact fitness (256). The order of increased fitness was WT HIV-1B > PYKE > PYQE > PTAPPd> HIV-1C WT. In addition, the drug sensitivity profile of HIV-1C viruses containing PYKE and PYQE had reduced susceptibility to LPV. One of the PYKE viruses also displayed reduced sensitivity to TAF. All the viruses displayed high to medium susceptibility to NNRTIs and INSTIs tested (EC_{50} fold change <2).

Conclusion:

PYxE insertion in HIV-1C provides a replication advantage that can affect the susceptibility of the virus to certain ARVs . Therefore, monitoring of patients with HIV-1C is crucial as therapy failure in the absence of known drug resistance mutations cases can be explained by patients that emerge with HIV-1C PYxE- carrying strains.

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PATHOGENESIS AND IMMUNITY

HIV-1 Subtype C with PYxE Insertion Has Enhanced Binding of Gag-p6 to Host Cell Protein ALIX and Increased Replication Fitness

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ABSTRACT Human immunodeficiency virus type 1 subtype C (HIV-1C) has a natural deletion of a YPxL motif in its Gag-p6 late domain. This domain mediates the binding of Gag to host cell protein ALIX and subsequently facilitates viral budding. In a subset of HIV-1C-infected individuals, the tetrapeptide insertion PYxE has been identified at the deleted YPxL motif site. Here, we report the consequences of PYxE insertion on the interaction with ALIX and the relevance regarding replication fitness and drug sensitivity. In our three HIV-1C cohorts, PYKE and PYQE were most prevalent among PYxE variants. Through *in silico* predictions and *in vitro* experiments, we showed that HIV-1C Gag has an increased binding to ALIX when the PYxE motif is present. To go more into the clinical relevance of the PYxE insertion, we obtained patient-derived *gag-pol* sequences from HIV-1C_{PYxEi} viruses and inserted them in a reference HIV-1 sequence. Viral growth was increased, and the sensitivity to the protease inhibitor (PI) lopinavir (LPV) and nucleoside reverse transcriptase inhibitor tenofovir alafenamide (TAF) was decreased for some of the HIV-1C PYxE variants compared to that of wild-type variants. Our data suggest that PYxE insertion in Gag restores the ability of Gag to bind ALIX and correlates with enhanced viral fitness in the absence or presence of LPV and TAF. The high prevalence and increased replication fitness of the HIV-1C virus with PYxE insertion indicates the clinical importance of these viral variants.

IMPORTANCE Genomic differences within HIV-1 subtypes is associated with various degrees of viral spread, disease progression, and clinical outcome. Viral budding is essential in the HIV-1 life cycle and mainly mediated through the interaction of Gag with host proteins. Two motifs within Gag-p6 mediate binding of host cell proteins and facilitate budding. HIV-1C has a natural deletion of one of these two motifs, resulting in an inability to bind to host cell protein ALIX. Previously, we have identified a tetrapeptide (PYxE) insertion at this deleted motif site in a subset of HIV-1C patients. Here, we report the incidence of PYxE insertions in three different HIV-1C cohorts, and the insertion restores the binding of Gag to ALIX. It also increases viral growth even in the presence of the antiretroviral drugs lopinavir and tenofovir alafenamide. Hence, PYxE insertion in HIV-1C might be biologically relevant for viruses and clinically significant among patients.

KEYWORDS ALIX, Gag, HIV-1, pathogenesis

Human immunodeficiency virus type 1 (HIV-1) is a global threat, with an estimated 36.9 million HIV-1-infected individuals and 940,000 deaths in 2017. Virulence of HIV-1 is determined by its capacity to replicate within infected cells and its ability to

Citation van Domselaar R, Njenda DT, Rao R, Sönnerborg A, Singh K, Neogi U. 2019. HIV-1 subtype C with PYxE insertion has enhanced binding of Gag-p6 to host cell protein ALIX and increased replication fitness. J Virol 93:e00077-19. https://doi.org/10.1128/JVI.00077-19.

Editor Viviana Simon, Icahn School of Medicine at Mount Sinai

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Received 16 January 2019
Accepted 7 February 2019
Accepted manuscript posted online 13 February 2019
Published 17 April 2019

infect new cells. Among different HIV-1 subtypes, more than 50% of all infections are caused by HIV-1 subtype C (HIV-1C), which is prevalent in South Africa, Zimbabwe, Mozambique, Botswana, Ethiopia, Eritrea, India, and in some parts of Brazil. However, HIV-1C has also become highly prevalent in several European countries, including Sweden (1, 2). The *gag* gene encodes a polyprotein that is proteolytically processed by viral and cellular proteases into six final products: p17 matrix protein (MA), p24 capsid protein (CA), spacer peptide 1 (SP1), p7 nucleocapsid protein (NC), spacer peptide 2 (SP2), and p6 protein (P6). These proteins are required for the assembly and release of new virions (3–6). The p6 late domain of Gag contains two conserved short peptide motifs that bind host factors to facilitate viral budding (6–8). The PTAP motif is present in both HIV-1 and HIV-2, and sometimes it is duplicated in HIV-1 genomes (9, 10). It mediates binding to ESCRT-I subunit tumor susceptibility gene 101 (TSG101) (11). The YPx_nL (where x_n represents a random number of any possible amino acids) motif binds to the ESCRT-III adaptor protein ALG-2-interacting protein X (ALIX) (12). However, this motif is deleted exclusively in HIV-1C (13). This deletion is associated with the loss of binding of p6 late domain to ALIX and a decrease in virus release from infected cells. Of note, HIV-2 lacks the YPx_nL motif but contains an alternative ALIX-binding motif, namely, PYKEVTEDL, that originates from simian immunodeficiency virus (SIV) in rhesus macaques (14).

Recently, we identified a tetrapeptide insertion, PYxE (where x represents a lysine [K], a glutamine [Q] or an arginine [R]), within the Gag protein in a subgroup of HIV-1C-infected individuals (C_{PYxEi} strains) (15). This C_{PYxEi} strain was preferentially identified in East African HIV-1C-infected patients but was less common among HIV-1C-infected patients from South Africa, India, and Germany (15). Furthermore, the PYxE insertion appears more frequent among patients on antiretroviral therapy, e.g., ritonavir-boosted protease inhibitors (PI), than in therapy-naive patients in India, and it is more frequent in therapy failure patients in South Africa (15, 16). Moreover, lower pretherapy $CD4^+$ T cell counts, higher plasma viral loads, and reduced increase in $CD4^+$ T cell counts were noted to be associated with PYxE insertion in HIV-1C patients compared to that of patients with wild-type HIV-1C (HIV-$1C_{WT}$) from East Africa (17). Furthermore, we observed that increased replication fitness in PYQE-inserted HIV-1C viruses was polymerase independent (17). A more recent study also claimed that Gag-protease is the major determinant of subtype differences in disease progression among HIV-1 subtypes (18).

As the tetrapeptide PYxE insertion was found at the site of the missing YPx_nL motif of HIV-1C Gag-p6, we hypothesized that this PYxE insertion restores the interaction of the Gag-p6 late domain with ALIX and thereby increases replication fitness and reduces sensitivity to PIs. A recent report showed that the PYRE insertion could rescue the viral growth of a PTAP-deleted HIV-1 variant, similar to the YPx_nL motif in the absence of a PTAP sequence (19). However, since the PTAP motif is always present in clinical HIV-1 isolates, it is not known if and how PYxE insertion affects HIV-1C pathogenicity in a clinically relevant context. To address this, we assessed the distribution and frequency of this PYxE insertion in different cohorts of HIV-1C-infected individuals. We also evaluated the ability of the PYxE motif to reconstitute the interaction of Gag with ALIX, its association with increased viral growth of clinical isolates, and its association with drug responses against all three drug classes: reverse transcriptase inhibitors (RTIs), PIs, and integrase strand transfer inhibitors (INSTIs).

(This article was submitted to an online preprint archive [20].)

RESULTS

Evolution of Gag-p6 late domain sequences in HIV-1B and HIV-1C. The sequence analysis of HIV-1B and South African and Indian HIV-1C Gag-p6 sequences (obtained from the Los Alamos Database) identified a conserved $LYPx_nL$ motif in HIV-1B, which was missing from HIV-1C (Fig. 1a). We noted a unique evolution of the lysine (K) residue in the HIV-1B and HIV-1C Gag-p6 late domain, which is the target of ubiquitination. All the amino acids positions are per the standard HXB2 coordinates.

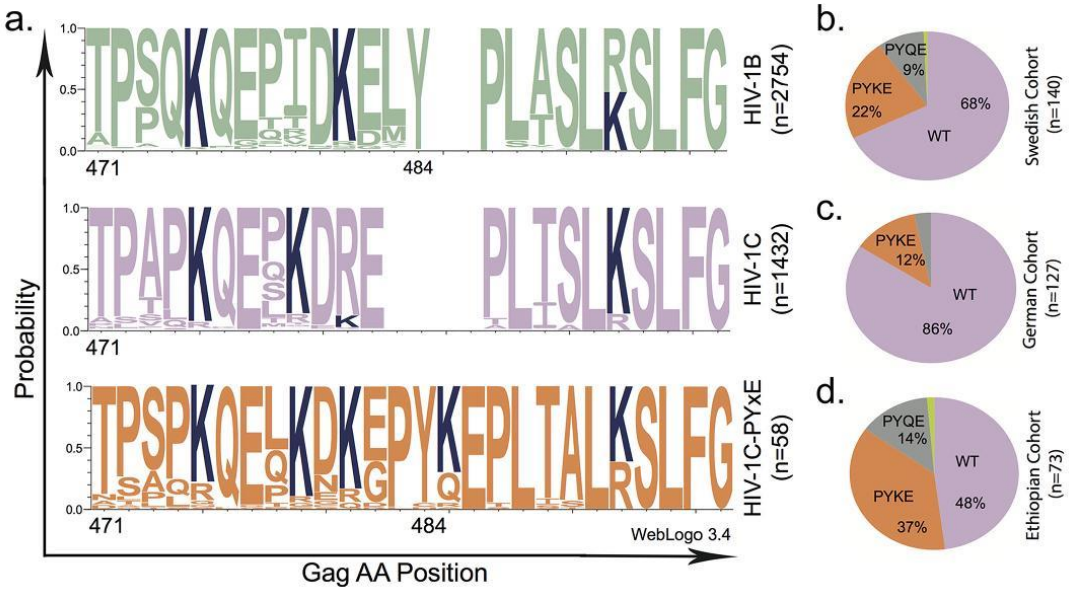

FIG 1 Consensus sequences and distribution of HIV-1 *gag*. (a) Aligned consensus sequences of *gag* from HIV-1 type B wild type (top), HIV-1 type C wild type (middle), and HIV-1 type C with a tetrapeptide insertion (bottom) from the Los Alamos database. Sequence logos with one-letter-coded amino acids were generated using WebLogo 3. (b to d) Distribution of HIV-1 type C with gag_{wt}, gag_{PYKE}, and gag_{PYQE} in patient cohorts from Sweden (b), Germany (c), and Ethiopia (d).

While the K475 residue was conserved in both HIV-1B and -C, there was an evolution of a lysine residue at amino acid position 479 along with K481R mutation in the majority of HIV-1C sequences. Interestingly, the multiple-sequence alignment of HIV-$1C_{PYxEi}$ strains identified conservation of the HIV-1C-specific K479 residue, but residue R481 in HIV-$1C_{WT}$ changed to a lysine residue within HIV-$1C_{PYxEi}$ strains and thus was identical to K481 in HIV-1B (Fig. 1a). When we analyzed Gag-p6 sequences from HIV-1C patients from the Swedish InfCare HIV cohort ($n = 140$), we found that 68% (95/140) of HIV-1C patients had a wild-type strain, whereas 32% (45/140) had a PYxE insertion; 22% (31/140) had a PYKE insertion, and 9% (13/140) had a PYQE insertion (Fig. 1b). All of the PYxE sequences in HIV-1C of patients from the Swedish cohort belonged to HIV-1C strains of Ethiopian and Eritrean origin. A similar trend was observed in the German HIV-1C cohort with 12% PYKE insertion (15/127) (Fig. 1c) and in the Ethiopian HIV-1C cohort with 37% (27/73) PYKE insertion (Fig. 1d). Thus, our analysis indicated that PYKE and PYQE are predominating in HIV-$1C_{PYxEi}$ viruses of all cohorts analyzed. Subsequently, we restricted our analysis to these two variants.

PYxE insertion enhances type C Gag: ALIX interaction. Before testing the interaction between Gag and ALIX, we evaluated the cellular localization of both proteins. 293T cells were cotransfected with green fluorescent protein (GFP)-tagged ALIX with or without three codon-optimized variants of mCherry-tagged HIV-1C Gag, namely, wild-type Gag or Gag that includes the tetrapeptide insertion PYKE or PYQE. Confocal analysis showed cytoplasmic localization of ALIX and predominant plasma membrane localization of all three Gag variants (HIV-$1C_{WT}$, HIV-$1C_{PYQEi}$, and HIV-$1C_{PYKEi}$) as the complete Gag produces virus-like particles (Fig. 2). Thus, the tetrapeptide insertion did not cause a change or defect in the localization pattern of Gag proteins at the plasma membrane.

Next, we evaluated the binding affinity of four HIV-1 Gag variants, HIV-$1B_{WT}$, HIV-$1C_{WT}$, HIV-$1C_{PYKEi}$, and HIV-$1C_{PYQEi}$, to ALIX (Fig. 3a). The late domain motifs'

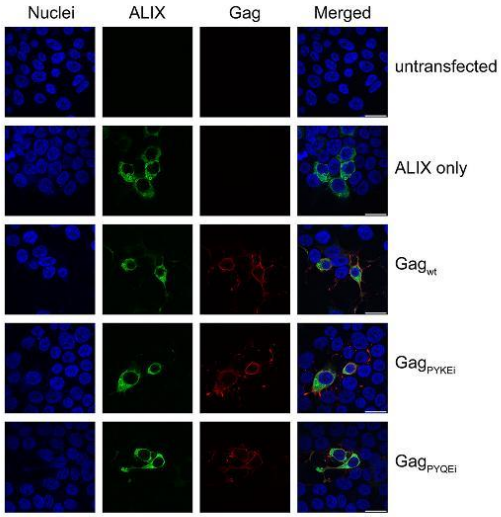

FIG 2 Cellular localization of Gag variants and ALIX. Shown are confocal images of HEK293T cells cotransfected with the GFP-tagged ALIX and mCherry-tagged HIV-1C Gag variants (wt, PYKEi, and PYQEi) expression plasmids. Nuclei are visualized in blue, ALIX in green, and Gag in red. Bars, 20 μm.

peptides were selected based on crystal structures of PDB entries 2XS1 and 2R02. The binding affinity of the Gag peptide with ALIX was statistically significantly higher in HIV-1B$_{WT}$ (32 $\pm$ 3 nM) than in any of the HIV-1C variants, i.e., HIV-1C$_{WT}$ (290 $\pm$ 6 nM), HIV-1C$_{PYKEi}$ (110 $\pm$ 4 nM), and HIV-1C$_{PYQEi}$ (96 $\pm$ 4 nM) (Fig. 3b). However, statistically significantly higher binding affinity to ALIX was observed with HIV-1C$_{PYKEi}$ and HIV-1C$_{PYQEi}$ than with HIV-1C$_{WT}$.

As the peptides may not retain their conformational structures, as in the complete protein, we confirmed the binding by coimmunoprecipitation pulldown assays. GFP-tagged ALIX and HIV-1C Gag variants were transfected either alone or together in 293T cells. A plasmid encoding HIV-1B Gag was used together with GFP-ALIX as a positive control for Gag-ALIX interaction. Lysates were prepared 24 h posttransfection, GFP-tagged proteins were pulled down, and coimmunoprecipitated proteins were analyzed by immunoblotting (Fig. 3c). Pulldown of GFP-tagged ALIX was efficient, and the amount of HIV-1B Gag was increased when it was cotransfected with GFP-ALIX, confirming that HIV-1B Gag binds to ALIX. The HIV-1C Gag wild type was also detected when cotransfected with GFP-ALIX, but HIV-1C Gag PYKE was more prominently detected. The intensity of HIV-1C Gag PYQE was consistently lower than that of the PYKE variant but slightly increased compared to that of wild-type HIV-1C Gag. Thus, the tetrapeptide insertion PYxE in the HIV-1C Gag-p6 late domain promotes binding of Gag toward ALIX.

***In silico* analysis predicts binding of ALIX to the PYKE motif in Gag facilitated by ubiquitination.** As reported before, the lysine residues in Gag-p6 are subjected to ubiquitination (21). We first predicted the likelihood of ubiquitination of the lysine residue of PYKE (K_{ins}) compared to that of lysine residues adjacent to the PYKE motif. Indeed, K_{ins} was predicted to be more likely ubiquitinated than lysine residues in the vicinity of the PYKE motif (Fig. 4a). Since the PYKE motif is present at the missing ALIX-binding site and contains three charged amino acids that can facilitate interaction with other proteins, we used the PYKE insertion (amino acids 483 to 486) in the molecular modeling to predict the interaction of the Gag-p6 late domain with ALIX. Amino acids E482, Y484, and E486 of Gag could directly facilitate binding to ALIX

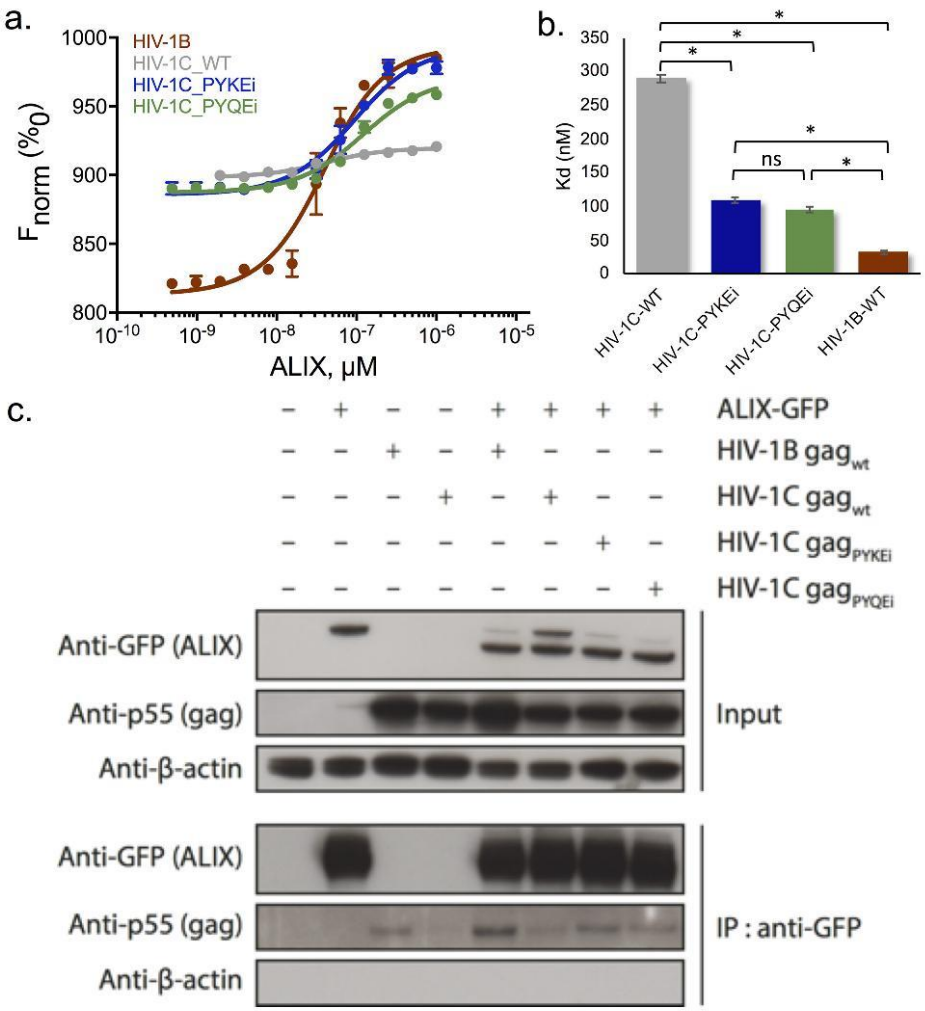

FIG 3 HIV-1C Gag with PYxE insertion has increased binding with ALIX. (a to d) Protein-peptide interaction using MST. The peptide sequences are given in the individual graphs. (e) The equilibrium dissociation constant (K_d) of the individual interactions. The smaller the K_d value, the greater the binding affinity of the ligand for its target. (f) HEK293T cells were cotransfected with GFP-tagged ALIX and Gag variants from HIV-1 type B or C. At 24 h posttransfection, cells were lysed and subjected to GFP pulldown. Input and pulldown samples were subjected to immunoblotting antibodies against GFP (ALIX), p55 (Gag), and β-actin. Data depicted are representative of at least two independent experiments.

(Fig. 4b and Table 1). Ubiquitin was predicted to bind K501 in ALIX and thereby affected the interaction between E486 in Gag-PYKE with K501 in ALIX. In addition, ubiquitin could also interact with E387 of ALIX and K_{ins} of Gag-PYKE through its residues K63 and E64, respectively. Thus, our *in silico* analysis predicted that the PYxE motif could restore the interaction of Gag with host cell ESCRT-III adaptor protein ALIX and that, in the case of a PYKE insertion, ubiquitination of the K_{ins} residue could further facilitate this interaction. We also tested the localization of ubiquitin by immunofluorescence. Since

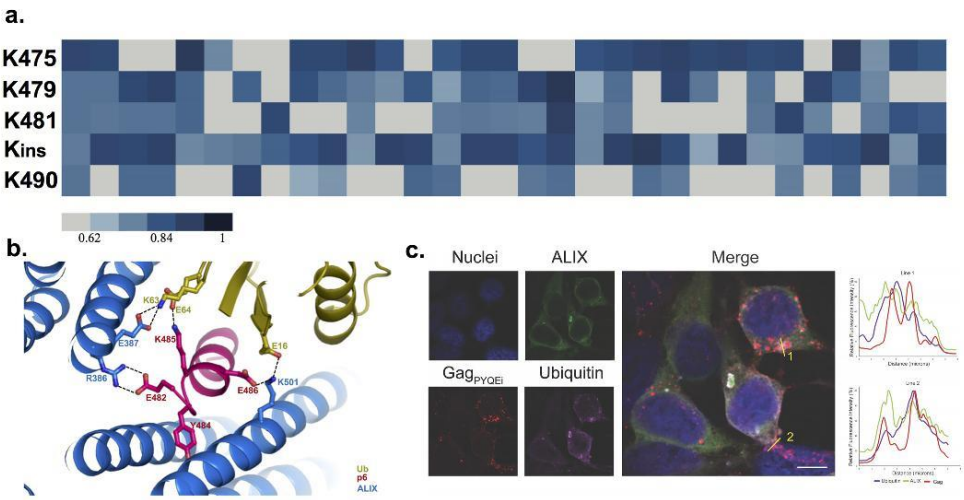

FIG 4 *In silico* binding prediction between PYKE inserted within HIV-1 type C Gag, ALIX, and ubiquitin. (a) Likelihood of ubiquitin binding to lysine residues in the vicinity of or within the PYKE motif. K_{ins} represents the lysine residue within the PYKE motif. (b) All proteins are presented as ribbon-like structures. The red ribbon represents Gag and its PYKE insertion, the blue ribbon ALIX and its Gag-binding sites, and the green ribbon ubiquitin. Amino acids involved in Gag-ALIX binding are shown in one-letter format. Interactions between amino acids are shown with black dotted lines. *In silico* docking was performed using Schrödinger software. (c) Confocal images of HEK293T cells transfected with the GFP-tagged ALIX and mCherry-tagged HIV-1C Gag PYQEi and HA-tagged ubiquitin expression plasmids. Nuclei are visualized in blue, ALIX in green, Gag in red, and ubiquitin in magenta. Bar, 20 μm.

both Gag and ALIX are known to be ubiquitinated, it was not surprising that ubiquitin colocalized with ALIX and Gag (Fig. 4c).

Differences in viral growth of clinical strains of HIV-1C with PYxE motif. Finally, we assessed the consequence of the PYxE insertion on HIV-1C replication fitness using clinical strains obtained from HIV-1C-infected patients. *gag-pol* sequences were cloned from one wild-type HIV-1C virus with a single PTAP motif and no PYxE insertion (PT01), three HIV-1C strains with a PYKE insertion (PT04-06), and one with a PYQE insertion (PT07). We also included two HIV-1C strains with a PTAP duplication (PT02 and PT03), as it has been shown to increase the viral fitness and affect the drug sensitivity (Fig. 5a) (22, 23). Then these sequences were cloned into the genome of a reference virus (NL4-3, HIV-1B wild type), replacing its *gag-pol* sequence. The various recombinant viruses were tested for viral replication in an *ex vivo* viral growth kinetic assay and compared to the reference virus in the MT-4 cell line (Fig. 5b) and primary $CD4^+$ T cells isolated from donor blood (Fig. 5c). In MT-4 cells, a statistically smaller amount of HIV-$1C_{WT}$ virus was observed in the supernatant than that for the HIV-$1C_{PYxEi}$ and

TABLE 1 List of amino acids that are involved in the interaction between Gag-P6, ALIX, and ubiquitin[a]

Interacting atoms	Residue 1	Residue 2	Distance (Å)	Interaction type
OE1–NH1	E482 (LD)	R386 (ALIX)	2.96	Charge-charge
OE2–NH2	E482 (LD)	R386 (ALIX)	2.83	Charge-charge
OE1–NZ	E387 (ALIX)	K63 (Ub)	3.05	Charge-charge
OE2–NZ	E387 (ALIX)	K63 (Ub)	2.97	Charge-charge
OE2–NZ	E64 (Ub)	K485 (LD)	2.93	Charge-charge
OE2–NZ	E486 (LD)	K501 (ALIX)	2.95	Charge-charge
OE1–NZ	E16 (Ub)	K501 (ALIX)	3.12	Charge-charge
OH–OD1	Y484 (LD)	D506 (ALIX)	2.72	Hydrogen bond

[a]E, glutamic acid; R, arginine; Y, tyrosine; D, aspartic acid; K, lysine; Ub, ubiquitin.

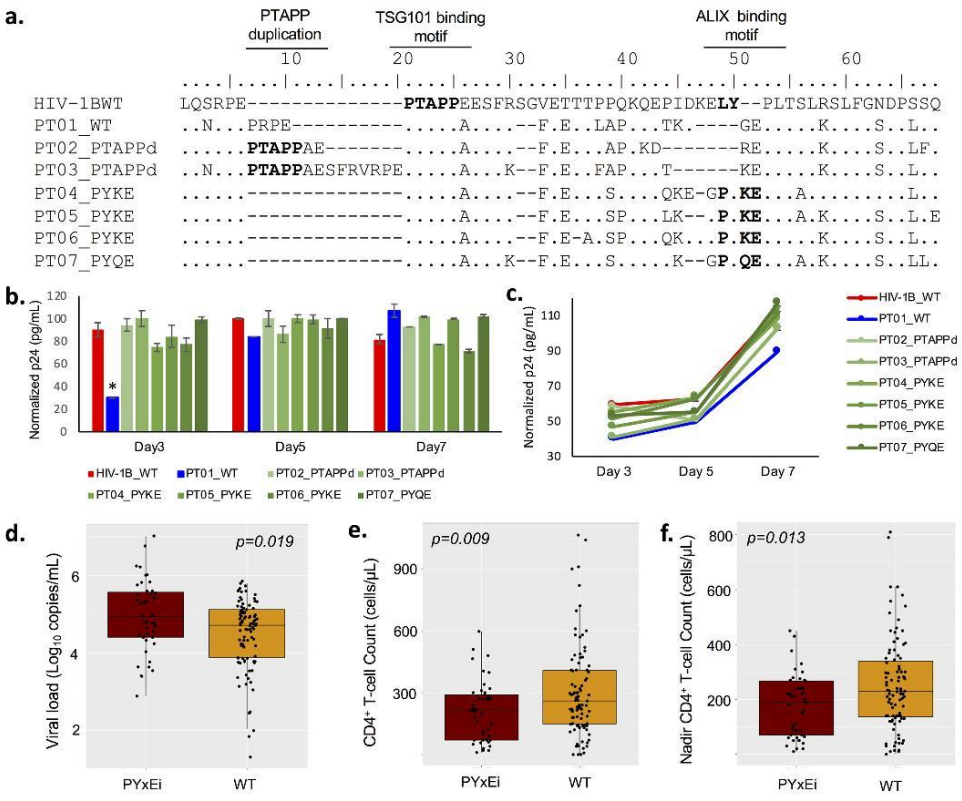

FIG 5 PYxE insertion increases viral growth and drug sensitivity toward protease inhibitor drug lopinavir. (a) *gag-pol* sequences were amplified from HIV-1 type C patient isolates and cloned into HIV-1 molecular clone pNL4-3. Recombinant viruses were propagated in HEK293T cells, and MOIs were determined in TZM-bl reporter cells. Viral growth assay was performed in MT-4 cells (b) or purified primary $CD4^+$ T cells (c). Cells were infected with the various recombinant viruses at an MOI of 0.05, and supernatants were harvested on days 0, 3, 5, and 7. Viral loads in supernatants were measured by HIV-1 p24 analysis on the HIV COBAS 8000 platform. Data represent the means ± standard deviations from triplicates. Clinical data regarding viral load at initiation of therapy (d), $CD4^+$ T cell count at initiation of therapy (e), and nadir $CD4^+$ T cell count (f) were collected from the Swedish HIV-1C cohort, and patients were grouped as HIV-$1C_{PYxEi}$-infected ($n = 45$) or HIV-$1C_{WT}$-infected individuals ($n = 95$).

HIV-1B reference viruses 3 days postinfection. At day 5 postinfection, the amount of HIV-$1C_{WT}$ virus was still reduced compared to those of the other viruses. The amount of HIV-$1C_{WT}$ in the supernatant was highest at day 7 postinfection. The reduction of HIV-$1C_{PYxEi}$ and HIV-1B viruses at day 7 postinfection is most likely caused by the death of the virus-producing cells, which was observed microscopically. Similar data were observed in $CD4^+$ T cells, where HIV-$1C_{WT}$, compared to all other viruses, showed the smallest amount of virus in the supernatant 7 days postinfection (Fig. 5c). Our data that viral replication for the HIV-1C wild-type clone was reduced compared to that of the HIV-1B reference virus are in concordance with a previous report (24). More importantly, our data showed that when a PTAP duplication or a PYxE insertion was present, viral growth was increased compared to that of the HIV-1C wild-type variant and was comparable to that of the HIV-1B wild type. This correlation suggests that HIV-1C strains with a PYxE insertion have a growth advantage compared to wild-type HIV-1C. This is

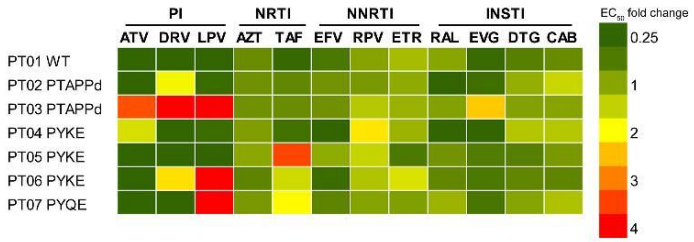

FIG 6 PYxE insertion increases drug sensitivity toward protease inhibitor drug lopinavir. TZM-bl reporter cells were infected with the individual recombinant HIV at an MOI of 0.05 and cultured in the presence of individual antiretroviral drugs at different dilutions. EC_{50} values for each virus with each drug were determined, and EC_{50} fold changes (FC) were calculated compared to those of the pNL4-3 reference virus. PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; INSTI, integrase strand transfer inhibitor; ATV, atazanavir sulfate; DRV, darunavir ethanoate; LPV, lopinavir; AZT, azidothymidine; TAF, tenofovir alafenamide; EFV, efavirenz; RPV, rilpivirine; ETR, etravirine; RAL, raltegravir; EVG, elvitegravir; DTG, dolutegravir; CAB, cabotegravir. The FC data presented in the heat map were the means from three individual experiments.

also in line with our earlier study that HIV-$1C_{PYQEi}$ viruses are more replication competent as well as more pathogenic (17).

We next checked whether the *ex vivo* data corroborate with the clinical data. In the Swedish HIV-1C cohort ($n = 140$), we checked the nadir $CD4^+$ T-cell count, $CD4^+$ T-cell count at the initiation of therapy, and viral load at initiation of therapy. The viral load was significantly higher in HIV-$1C_{PYxEi}$-infected individuals than in the individuals who were infected with wild-type HIV-1C viruses (Fig. 5d). The $CD4^+$ T-cell count at the initiation of therapy (Fig. 5e) and nadir $CD4^+$ T-cell count (Fig. 5f) were also statistically lower in the HIV-$1C_{PYxEi}$-infected individuals than in individuals infected with wild-type HIV-1C viruses. These data further strengthen our *ex vivo* findings.

Effect of PYxE strains in susceptibility toward antiretroviral drugs. Several reports have shown that a PYxE insertion is more frequent among HIV-1C therapy failure patients (16, 25). To examine whether recombinant viral clones without any known drug resistance mutations to RTIs, PIs, and INSTIs responded differently to various antivirals, the target cells were infected with individual viral clones in the presence of a specific drug. The viral replication was assessed, and the 50% effective concentration (EC_{50}) was calculated and fold change of the EC_{50} was noted (Fig. 6). No differences in EC_{50} values were observed among the various recombinant viral clones in the presence of the nonnucleoside RTIs (NNRTIs) efavirenz (EFV), rilpivirine (RPV), and etravirine (ETR) or the INSTIs raltegravir (RAL), elvitegravir (EVG), dolutegravir (DTG), and cabotegravir (CAB).

In contrast, with the PI lopinavir (LPV), one out of two viral clones with a PTAP duplication and two out of four with PYxE insertion showed a >4-fold decreased sensitivity toward the drug. However, there was no change in susceptibility against DRV and ATV. One of the other viral clones with a PYxE insertion showed a 3.5-fold decreased sensitivity to the nucleoside reverse transcriptase inhibitor (NRTI) tenofovir alafenamide (TAF). Thus, our data indicate that PYxE insertion alters sensitivity to the PI LPV and NRTI TAF, which may be due to the increased replication fitness. However, this is not universal across all the HIV-1_{PYxEi} strains and could be due to coevolution of other amino acids in the Gag protein.

DISCUSSION

Genetic diversity among HIV-1 strains and their adaptation can provide advantages for individual viral strains in different biological contexts. Here, we described the distribution of HIV-1C containing tetrapeptide PYxE insertion within Gag and assessed the binding efficiency of Gag with the host cell protein ALIX. Our results showed that

the PYxE insertion enhances the binding capacity of HIV-1C Gag to ALIX in a potentially ubiquitin-dependent or -independent manner. Also, the insertion increased the replication fitness of the virus in both an *in vitro* model using a T cell line as well as in primary $CD4^+$ T cells and seemed to alter the sensitivity against the PI LPV and NRTI TAF in some of the strains.

Although the PYxE insertion is quite prevalent among HIV-1C from Ethiopian and Eritrean individuals, it is not as prevalent in South African or Indian HIV-1C strains (16, 25). The 12-bp sequence encoding the PYxE motif is not present in non-subtype C M group HIV-1 sequences but is present within the Gag-p6 late domains of SIV_{mac239}, SIV_{smE543}, and their close relative HIV-2 (14, 26). As HIV-1C has a natural deletion of the YPx_nL motif and insertion of PYxE has replication advantages, we hypothesize that these strains have evolved through a recombination event of HIV-1C with either SIV or HIV-2. It is well described that the HIV-1 epidemic in Ethiopia geographically clusters very strongly (27), and the Ethiopian HIV-1C has been proposed to originate from either a single lineage or multiple descendants (28). However, to explain why the PYxE insertion is significantly more prevalent in HIV-1C from Ethiopia and Eritrea than from South African and Indian strains needs a further evolutionary study to prove this hypothesis.

The Gag-p6 late domain mediates viral budding through the PTAP motif, the YPx_nL motif, and the PYxE motif. The PTAP motif mediates binding of Gag to ESCRT protein TSG101 and is present in all HIV and SIV variants. Therefore, this domain appears to be necessary for viral budding. Although one report shows that PTAP duplication only increases HIV-1 replication in the presence of a PI (23), we and others observed increased viral growth when HIV-1 contained a PTAP duplication (22). Although deletion of the PTAP motif has not been identified in clinical strains so far, artificial deletion or mutation of this PTAP motif severely abrogates release of HIV-1 from infected cells (11, 29, 30). However, the presence of either one of the two other motifs that both facilitate binding to host cell protein ALIX still allows for virion release (12, 14, 19, 31, 32). This indicates that both TSG101- and ALIX-mediated pathways for viral budding are not dependent on each other, and each pathway is sufficient to mediate viral budding. The first ALIX-binding motif is YPx_nL and is present in most HIV-1 subtypes but not in HIV-1C or HIV-2 (13, 14). HIV-2 Gag can mediate binding to ALIX through a distinct PYKEVTEDL motif, and mutations in this motif can abrogate ALIX binding and decrease virion release (14, 26). Thus, the PYxE motif in a subgroup of HIV-1C strains is similar but shorter than the ALIX-binding motif found in HIV-2. We showed that this insertion of PYxE in HIV-1C Gag at the natural deletion site of the YPx_nL domain could reconstitute binding of HIV-1C Gag to ALIX. Hence, together with our observed PYxE insertion in HIV-1C, all HIV-1 subtypes have an ALIX-binding motif next to their PTAP motif within the Gag late domain.

Our data suggest that ubiquitin plays a role in facilitating the binding of ALIX to the PYKE motif. This is not surprising, as ubiquitin has been shown to be involved in viral budding of HIV. Although dispensable for virus budding, ubiquitination of Gag and the ESCRT proteins is essential for efficient release of HIV-1 from infected cells (21, 33, 34). Ubiquitination of the lysine of the PYKE motif could increase the binding between ALIX and Gag and thereby mediate more efficient viral budding. Indeed, coimmunoprecipitation of HIV-1C Gag-PYKE was more efficient than that of the Gag-PYQE variant. As the PYKE motif insertion seems to be the original insertion within HIV-1C Gag, ubiquitination of its lysine might not be essential, since other variants of the PYKE motif, i.e., PYQE and PYRE, are identified among HIV-1C strains along with the evolution of another lysine residue in the late domain. Because we have only a few clinical strains with a PYxE insertion, we could not identify differences in viral growth between HIV-1C Gag-PYKE and other PYxE variants. Nonetheless, whereas the other three residues seem to be required for the binding of ALIX to Gag, the lysine residue might provide increased stability for this interaction.

Although the ALIX-mediated viral budding mechanism appeared to be lost in HIV-1C, it is surprising to observe that HIV-1C PYxE strains were prevalent among

HIV-1C-infected individuals. This suggests that the interaction of Gag with ALIX is of biological importance for the virus. In our limited cohort of HIV-1C-infected patients, PYxE insertion was always copresent with the PTAP motif. This suggests that the PYxE motif is not redundant from the PTAP motif-mediated viral budding process through TSG101 or a compensatory mechanism for loss of the PTAP motif, and HIV-1 requires both motifs for efficient viral replication. Importantly, viruses with HIV-1C Gag PYKE or PYQE insertion showed enhanced viral growth compared to viruses with HIV-1C wild-type Gag (Fig. 5). This finding is in line with a recent report that showed that a PYRE insertion within Gag enhances HIV-1C viral growth (19).

Decreased sensitivity toward PI LPV or NRTI TAF was found in several HIV-1C strains with a PTAP duplication or PYxE insertion. For the PTAP duplication, it has previously been reported that it could enhance HIV-1 growth in the presence of the PIs lopinavir and ritonavir (23). For the PYxE insertion, our earlier studies have shown that the PYxE insertion is more frequent in HIV-1C therapy failure patients (15, 16). Nonetheless, since our clinical HIV-1C PYxE strains did not have any known drug resistance mutations, it seems likely that the correlation of these viruses with advanced viral growth and reduced sensitivity to the PIs LPV and TAF could increase the risk for therapy failure in HIV-1C PYxE-infected patients. Mechanistic studies show that at the time of reverse transcription of HIV-1C from the RNA template, the reverse transcription favors pausing at the nucleotides in the HIV-1C K65 position (AAG), and this correlates with an increased probability for the development of tenofovir (TDF)/TAF mutation K65R (35, 36). However, no studies have shown any corelation with Gag mutation on the susceptibility of TAF. We are presently performing research to understand the role of Gag mutations on sensitivity for TAF, as both TDF and TAF are used globally.

In conclusion, our study showed that PYxE insertion in HIV-1C of patients originating from Ethiopia and Eritrea restored the interaction of Gag with ALIX, which is mediated in a ubiquitin-dependent or -independent way. Importantly, the insertion was positively correlated with the replication fitness that could affect the sensitivity against LPV in the absence of any PI drug resistance mutation. Based on the present study, our earlier clinical studies (15–17), and the study by Chaturbhuj et al. (19), we posit that a PYxE insertion in HIV-1 subtype C strains provides a replication advantage that can affect the susceptibility of the virus to certain antiretroviral drugs. As the PYxE insertion evolved within treatment failure patients infected with HIV-1C from both India and South Africa, HIV-1C_{PYxEi} also provides a replication advantage following treatment failure and following evolution of drug resistance mutations that may cost replication fitness. Most importantly, reduced sensitivity against TAF in the absence of any TAF mutation needs further studies to analyze any role of Gag mutations in reducing susceptibility to TAF. Altogether, it will be important to follow up on whether HIV-1C_{PYxEi} strains are emerging following the failure of antiretroviral therapy with LPV- and TAF-based regimens due to increased replication fitness. This information could provide important insights into the clinical significance of the PYxE insertion within HIV-1C Gag.

MATERIALS AND METHODS

Cell culture and plasmids. Cells were cultured in 5% CO_2 at 37°C. HEK293T cells were maintained in Dulbecco's modified Eagle medium (DMEM; Sigma, USA) supplemented with 10% fetal calf serum (Sigma, USA), 2 mM L-glutamine (Sigma, USA), 0.1 mM MEM nonessential amino acids (Gibco/Thermo Fisher Scientific, USA), and 10 U/ml penicillin combined with 10 μg/ml streptomycin (Sigma). TZM-bl reporter cells were also maintained in the medium mentioned above. This reporter cell line is a derivative of HeLa cells, but it stably expresses high levels of CD4 and CCR5 on the cell surface and has integrated copies of the luciferase gene under the control of the HIV-1 promoter that allows simple and quantitative analysis of HIV-1 infection. MT-4 cells were maintained in RPMI 1640 (Sigma, USA) medium supplemented with 10% fetal calf serum, 20 U/ml penicillin, and 20 μg/ml streptomycin. Cells were transfected using FuGene HD according to the manufacturer's instructions (Promega, USA) in a 3:1 ratio with DNA.

The pEGFPN1 and mCherry plasmids were obtained from Addgene. The plasmid encoding hemagglutinin (HA)-tagged ubiquitin was a kind gift from Soham Gupta (Karolinska Institutet, Sweden). pCR3.1/HIV-Gag-mCherry (HIV-1B Gag) and pCR3.1-GFP-ALIX plasmids were kind gifts from Paul Bieniasz (The Rockefeller University). Codon-optimized HIV-1 Gag with a PYQE motif gene of East African HIV-1C viruses was cloned into the pCR3.1/HIV-Gag-mCherry plasmid that contained an mCherry protein C-terminal tag sequence. The Gag-PYQE-mCherry plasmid was modified by site-directed mutagenesis to

make a base pair substitution mutation (C to A) that changes the PYQE motif to PYKE using the Q5 SDM kit (New England Biolabs, USA). Gag-PYQE-mCherry plasmid was linearized by PCR using 5′-AAGGAGC CTCTGACGAGCC-3′ as the forward primer (with the C-to-A base pair substitution underlined) and 5′-ATAGGGACCCTGGTCTTTCAGC-3′ as the reverse primer. Linear products were recircularized using DpnI, a polynucleotide kinase, and a ligase from the SDM kit by following the manufacturer's protocol. DNA sequences were verified by Sanger sequencing.

Gag-p6 sequences and clinical specimens. To identify the distribution of PYQE, PYRE, and PYKE motifs, we reanalyzed the HIV-1C Gag-p6 sequences that were collected from three cohorts, the Swedish InfCare Cohort ($n = 140$), the German Cohort ($n = 127$), and the Ethiopian Cohort ($n = 73$), as reported previously, with respect to naturally occurring polymorphisms in the PYxE motif (15). Additional HIV-1B ($n = 2,754$) and HIV-1C ($n = 1,432$) Gag-p6 sequences were collected from the Los Alamos HIV-1 Database. Stored plasma samples from therapy-naive patients infected with HIV-1C ($n = 10$) were randomly selected based on Gag-p6 sequences from the HIV cohort at Karolinska University Hospital, Stockholm, Sweden.

Recombinant virus production with patient-derived *gag-pol*. The recombinant viruses were produced as described by us recently (37). Briefly, the *gag-pol* fragment (HXB2:0702-5798) was cloned into the pNL4-3 plasmid following digestion with BssHII and SalI (New England Biolab, USA) and ligation using T4 DNA ligase (New England Biolabs, USA). The chimeric viruses were produced by transient transfection of the plasmids into the 293T cell line using FuGene HD and harvested 72 h later by a collection of the cell-free supernatant cleared by centrifugation and stored in aliquots at −80°C.

***In silico* analysis: molecular modeling, docking, and ubiquitin binding motif prediction.** Homology modeling techniques generated the structure of HIV-1C Gag late domain with the PYKE insertion. The published crystal structures of the Gag late domain in complex with ALIX (PDB entries 2XS1 [14] and 2R02 [38]) were used as template molecules to model the HIV-1C Gag late domain. For this purpose, the Prime utility of Schrödinger Suite (Schrödinger Inc., USA) was used. The structure was subjected to restricted minimization using OPLS_2005 force field. The resulting structure of the HIV-1C Gag late domain structure was docked into the crystal structure of ALIX (PDB entry 2XS1) using the PIPER protein-protein docking program of BioLuminate Suite (Schrödinger Inc., USA) after deleting the SIV_{mac239} PYKEVTEDL late domain structure. The resulting structure was further minimized for 1,000 iterations to remove steric clashes. A similar protocol was used to form the complex among ALIX, Gag late domain, and ubiquitin. The crystal structure of ubiquitin in complex with the TSG101-binding PTAP domain (PDB entry 1S1Q) was used after deleting TSG101 coordinates (39). The entire complex was then subjected to molecular dynamics simulations for 1,000,000 steps with a step size of 50 ps using an OPLS3 force field. The most energetically stable model of the complex was used for further analysis. To obtain energetically favored polar interactions, the conformational search of polar side chains at the interface of proteins was also conducted.

The prediction of ubiquitin binding with the HIV-1C Gag late domain with the PYKE motif P6 was performed by adding the amino acid sequence in the prediction tools presented at the website http://www.ubpred.org/.

Immunofluorescence. HEK293T cells were cultured on poly-L-lysine-coated glass coverslips and cotransfected with GFP-tagged ALIX and mCherry-tagged codon-optimized Gag variants. At 24 h posttransfection, cells were washed twice with phosphate-buffered saline (PBS), fixed in 10% formalin (Sigma, USA) for 20 min at room temperature, washed three times with PBS, and stored in PBS at 4°C. Later, nuclear counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) was performed, followed by mounting of the glass coverslips on glass slides. When HA-ubiquitin was also cotransfected, cells were fixed in ice-cold methanol for 15 min at −20°C, washed twice with PBS, and stored in PBS at 4°C. For ubiquitin detection, fixed cells were incubated with the anti-HA antibody (mouse monoclonal, clone 12CA5; Sigma, USA) for 1 h at room temperature, followed by goat-anti-mouse-Alexa Fluor 647-Plus-conjugated secondary antibody (Invitrogen, USA) for 1 h at room temperature before nuclear counterstaining with DAPI. Fluorescence was analyzed by confocal laser scanning microscopy using a Nikon single-point scanning confocal microscope with 60×/1.4-numeric-aperture oil objective (Nikon, Japan). Fluorescence intensity was measured (along drawn lines) using Fiji/ImageJ software (40). The highest fluorescence intensity value for each fluorophore along each line was set to 100%, and values were plotted as relative fluorescence intensity in percentage against distance in microns using GraphPad Prism v6 (GraphPad, Inc., USA).

MST. The microscale thermophoresis (MST) experiments were conducted with a Monolith NT0.115 instrument (NanoTemper Technologies) at 40% MST and LED power at 25°C. Hexahistidine-tagged ALIX protein was labeled by nitrilotriacetic (NTA) dye (NanoTemper Technologies) using the manufacturer's protocol. The peptides were synthesized at the Molecular Interaction Core (University of Missouri). Thermophoresis was induced by mixing peptides (0.001 to 1 μM) and fixed NTA-labeled ALIX (50 nM) in a buffer of 50 mM Tris-Cl, pH 7.8, 100 mM NaCl, and 0.1% pluronic-F127. The binding isotherms were obtained by plotting the difference in normalized fluorescence against increasing peptide concentration. The binding affinities were determined to fit the data points to a quadratic equation (equation 1) using nonlinear regression with MO Affinity software (NanoTemper Technologies), Prism (version 6.0; GraphPad, Inc.), or OriginLab (version 18; OriginLab Corp., Northampton, MA, USA).

$$F_b = \frac{\left(K_{d1} + [\mathrm{PEP}_0] + [\mathrm{ALIX}_0]\right) - \sqrt{\left(K_{d1} + [\mathrm{PEP}_0] + [\mathrm{ALIX}_0]\right)^2 - 4[\mathrm{ALIX}_0][\mathrm{PEP}_0]}}{2[\mathrm{ALIX}_0]} \quad (1)$$

Where F_b is the fraction of ALIX/peptide complex, K_d = [ALIX][PEP]/[ALIX-PEP], [ALIX] is the concentration of free ALIX, [$ALIX_0$] is the concentration of total ALIX, [PEP] is the concentration of free peptide, and [PEP_0] is the total concentration of peptide.

Coimmunoprecipitation assay. HEK293T cells were cotransfected with GFP-tagged ALIX and codon-optimized Gag variants from HIV-1B or HIV-1C. At 24 h posttransfection, cells were collected and washed twice with ice-cold PBS, and immunoprecipitation of GFP-tagged proteins was performed using a GFP-Trap_A kit (Chromotek, Germany) according to the manufacturer's protocol. Briefly, cells were lysed in ice-cold lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, supplemented with a protease cocktail inhibitor from Roche [Switzerland]) for 30 min on ice. Lysates were diluted in ice-cold wash buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA) supplemented with a protease cocktail inhibitor from Roche, input samples (10% of total) were saved for immunoblot analysis, and the remaining (90%) part of the diluted lysates was incubated with equilibrated GFP-Trap_A beads end over end for 1 h at 4°C. Beads were washed three times with ice-cold wash buffer before resuspending in 2× Leammli buffer (4× buffer diluted 1:1 with PBS; Invitrogen) and heated for 10 min at 95°C. The protein concentration of input samples was determined by DC protein assay (Bio-Rad, USA) according to the manufacturer's microplate assay protocol. Equal amounts of protein for input samples and equal volumes of pulldown samples were subjected to immunoblotting using primary antibodies against GFP (rabbit monoclonal, clone EPR14104; Abcam, UK), Gag (rabbit polyclonal to HIV1 p55 + p24 + p17; ab63917; Abcam), and β-actin (rabbit polyclonal; ab8227; Abcam) and secondary horseradish peroxidase-conjugated antibodies (polyclonal goat anti-rabbit; Dako/Agilent, USA). Immunoblotted proteins were detected using the enhanced chemiluminescence detection system (Pierce/Thermo Fisher Scientific, USA) and Hyperfilms (Amersham/GE Healthcare Life Sciences, UK).

Viral growth kinetics assay in MT-4 cells. First, the 50% tissue culture infective dose ($TCID_{50}$) for each propagated recombinant viral clone was determined in TZM-bl reporter cells. For each recombinant viral clone, 10-fold serial dilutions were prepared to range from 10^2 to 10^7 in dilution factor from a single aliquot in medium containing DEAE (20 μg/μl). TZM-bl cells were plated in 96-well plates, and six replicates were incubated with the various virus dilutions for 48 h at 37°C in a 5% CO_2 humidified incubator. Virus infectivity was quantified by measuring Renilla luciferase activity (relative light units [RLU]) using a Bright-Glo Luciferase assay system (Promega, USA) on a Tecan microplate reader (Tecan Infinite 200 Pro; Tecan Group Ltd., Switzerland). The Spearman-Karber method was used to calculate the $TCID_{50}$ for each recombinant viral clone. MT-4 cells (2×10^5) then were seeded in a 12-well plate and infected with each recombinant virus at a multiplicity of infection (MOI) of 0.05 PFU per cell in triplicates for each condition. Supernatants were collected 0, 3, 5, and 7 days postinfection, and HIV-1 p24 levels in supernatants were measured on the HIV COBAS 8000 platform (Roche Diagnostics, Switzerland). Viral growth kinetics (VGK) were analyzed as a relationship between the signal-to-cutoff ratio (i.e., electrochemiluminescence signal of the sample relative to calibrated negative samples for the HIV combi PT kit on the COBAS 8000 system) for each time point and replicate. Graphical representation of the result was made using GraphPad Prism, v6 (GraphPad Inc., USA).

Isolation of $CD4^+$ T cells. Primary $CD4^+$ T cells were isolated from donor peripheral blood mononuclear cells (PBMCs). Briefly, 4.3×10^8 PBMCs were obtained from 50 ml buffy coat using the standard method of gradient separation using Ficoll Histopaque reagent followed by centrifugation. Isolated PBMCs were used to isolate $CD4^+$ T cells by negative selection using the EasySep human $CD4^+$ T-cell isolation kit (Stem Cell Technologies, Canada). Briefly, PBMCs were pooled in 5 ml polystyrene round-bottom tubes at a concentration of 5×10^7 cells and incubated with 50 μl of an antibody cocktail for 5 min at room temperature before mixing with RapidSphere beads followed by magnetic separation to yield pure $CD4^+$ T cells.

Viral growth kinetics assay in $CD4^+$ T cells. $CD4^+$ T cells were cultured using RPMI medium supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. The cells were stimulated with phytohemagglutinin (20 μg/ml final concentration) for 3 days before starting the assay. To start the viral growth kinetics assay, 1×10^6 $CD4^+$ T cells were seeded in each well in a V-bottom 96-well culture plate and infected with viruses at an MOI of 0.05 PFU/cell in the presence of 20 μg/ml of DEAE. The plate was spinoculated at 37°C for 2 h at 800 rpm in a temperature-controlled centrifuge. After that, the plate was further incubated in a 5% CO_2 incubator for 8 h. Afterward, infected cells were transferred to a 48-well culture plate and washed six times before harvesting the initial supernatant from each corresponding well and designating it day 0. The experiment was then monitored for 7 days, and the supernatant was harvested on days 3, 5, and 7. HIV p24 was then measured in the supernatants using an HIV alliance p24 enzyme-linked immunosorbent assay kit (Pekin Elmer, USA), and results were analyzed using GraphPad Prism, v6 (GraphPad, Inc., USA).

DSA. The following drugs were purchased from Selleckchem, USA: atazanavir sulfate (ATV), darunavir ethanoate (DRV), lopinavir (LPV), azidothymidine (AZT), tenofovir alafenamide (TAF), efavirenz (EFV), rilpivirine (RPV), etravirine (ETR), raltegravir (RAL), elvitegravir (EVG), dolutegravir (DTG), and cabotegravir (CAB). The drug sensitivity assay (DSA) was performed by determining the extent to which the antiretroviral drug inhibited the replication of the reference virus (pNL4-3) and wild-type, PTAPP-duplicated, and PyxE-derived recombinant viruses. One round was required for all drugs, as previously reported (37), except for the PI drugs, which required two rounds of infection. Briefly, for the two-round infection using PI DSA, TZM-bl cells (1×10^4) were seeded in 96-well plates and cultured for 24 h. Drugs were serially diluted in culture media (ranging from 0.1 μM to 0.01 pM) and added in triplicate to the cells. The following day, viruses were added to each well at an MOI of 0.05 PFU/cell in the presence of DEAE (10 μg/ml). After 48 h, the viral supernatant from respective 96-wells plates with each drug was transferred to newly TZM-bl preseeded 96-wells plates without adding new drugs. After that, virus

replication was quantified by measuring Renilla luciferase activity (in RLU) using the Bright-Glo Luciferase assay system (Promega, USA) 48 h after reinfection. Drug concentrations required for inhibiting virus replication by 50% (EC_{50}) were calculated by a dose-response curve using nonlinear regression analysis (GraphPad Prism, version 6.07; GraphPad Software, USA). The DSA experiments were performed with three technical replicates for each virus with the specified dynamic concentration range of the drug, and at least two independent analyses were performed. The reproducibility of the DSA was assessed by the 95% confidence interval obtained for the drug EC_{50} and the degree of correlation between technical replicates. The output for the drug EC_{50} results was used to compute the fold change value (FCV) for each virus relative to pNL4-3.

Ethical consideration. The study was approved by regional ethics committees of Stockholm (2014/928–31 and 2005/1167–31/3). The patient information was anonymized and de-identified prior to analysis.

ACKNOWLEDGMENTS

The study was funded by grants from the Swedish Research Council (2017-01330, U.N.; 2016-01675, A.S.), Karolinska Institutet Doctoral Student Funding (KID2015-154, U.N.), and the Stockholm County Council (ALF 20160074, A.S.). We thank S. G. Aralaguppe, W. Zhang, and S. Svensson Akusjärvi for technical assistance. The microscopic part of the study was performed at the Live Cell Imaging Facility, Karolinska Institutet, Sweden, supported by grants from the Knut and Alice Wallenberg Foundation, the Swedish Research Council, the Centre for Innovative Medicine, and the Jonasson Center at the Royal Institute of Technology, Sweden.

The following reagents were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl cells (number 8129) from John C. Kappes and Xiaoyun Wu, MT-4 cells from Douglas Richman, and HIV-1 NL4-3 infectious molecular clone (pNL4-3) from Malcolm Martin (number 114).

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5.2 PAPER II

Article title

Antiretroviral potency of 4'-Ethnyl-2'-Fluoro-2'-deoxyadenosine, Tenofovir alafenamide and second-generation non-nucleoside reverse-transcriptase inhibitors across diverse HIV-1 subtypes

Publication: The Journal of Antimicrobial chemotherapy (JAC)

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Author Contribution and declaration

U. N. conceived and designed the studies and analysis plan. D. T. N. and S. G. A. performed the virological assays (supervised by U. N.). D. T. N., S. G. A. and U. N. are responsible for the virological data. D. T. N. performed all the statistical analysis for the virological assays and prepared the figures. K. S. and R. R. performed the biochemical assays. K. S. wrote the biochemical part of the study. K. S. and S. G. S. are responsible for the biochemical data. A. S. interpreted the clinical data and is responsible for the InfCare cohort. D. T. N. wrote the first draft of the manuscript, which was reviewed by U. N., K. S., A. S. and S. G. S. All the authors approved the final version of the manuscript

Background: the goal of **paper II** was to understand and describe any HIV-1 subtype-specific differences to the response of clinically approved and newer ARV drugs. The specific aim investigated the *ex vivo* potency of the novel drug EFdA in comparison with first and second-generation NNRTIs – NVP, EFV and ETR, RPV respectively. In addition, TAF was also included as an approximate comparator of the same class to EFdA.

Main Findings: The results obtained indicated that EFdA had a superior *ex vivo* potency compared to all other drugs used in the study in inhibiting all the recombinant viruses (n=24) tested independent of HIV-1 subtype. EFdA also inhibited RTI resistant viruses (n=9) better

than TAF. Independent subtype inhibition of EFdA, was also confirmed by steady-state kinetic experiments that showed no difference in the incorporation efficiency of different phosphorylated forms of EFdA by different HIV-1 reverse transcriptase (RT) subtypes (01_AE, 02_AG, C and B). HIV-1 subtype representation for recombinant viruses used in DSA experiments included HIV-1B (n=6),) and HIV-1 A-like (02AG, 01_AE and A1 (n=4)) and HIV-1C (n=14). However, DSA data showed HIV-1C to have a significantly reduced susceptibility to RPV and ETR ($p = 0.017$ and $p= 0.004$ respectively). The *in vitro* DSA data correlated well with the biochemical experiments performed in the study. More specifically, biochemical data was able to demonstrate molecular differences of the inhibitory potential of NNRTI drugs mentioned above, against different HIV-1 RT subtypes. HIV-1C RT was also shown to have reduced susceptibility to ETR and RPV compared to other HIV-1 non-C subtype RTs.

Conclusion: EFdA inhibits both wild-type and RTI-resistant viruses efficiently in a subtype independent manner. Follow up research is required to understand the impact of RPV DRM in non-B subtypes

5.4 PAPER III

Article title

Ex-vivo antiretroviral potency of newer integrase strand transfer inhibitors cabotegravir and bictegravir in HIV type 1 non-B subtypes Publication: The Journal of Antimicrobial chemotherapy

Publication: AIDS

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Author Contribution and declaration

UN, KS and AS conceived and designed the studies and analysis plan. DTN. and SGA. performed the virological assays (supervised by UN.). DTN., SGA. and UN. are responsible for the virological data. DTN. performed all the statistical analysis for the virological assays and prepared the figures. RCL performed the biochemical assays. KS. wrote the biochemical part of the study. KS. and SGS. are responsible for the biochemical data. AS. interpreted the clinical data and is responsible for the InfCare cohort. UN wrote the first draft of the manuscript. All the authors approved the final version of the manuscript

Background: The observation of subtype-specific differences in paper II with the use of drugs from the RTI class made it rational to continue the investigation of subtype-specific differences in the INSTI class. This was the primary goal of **paper III**. Recombinant viruses (n=24) described in paper III were used to investigate the *in vitro* drug sensitivity of first (RAL, EVG) and second-generation (DTG, CAB, BIC) INSTI drugs.

Main Findings: The DSA results obtained indicated that second-generation INSTIs have a subtype-independent *in vitro* potency except for DTG, which indicated to be more potent in non-B compared to HIV-1B subtypes. To add on, DTG, BIC, and CAB performed well in

inhibiting A-like viruses compared to HIV-1C and B Subtypes. However, HIV-1C and HIV 02_AG had a significantly reduced susceptibility against first-generation drug EVG ($p < 0.05$). RAL displayed the least potency against all subtypes tested. DSA data also compared very well with integrase strand-transfer activity biochemical experiments.

The secondary goal in **paper III** investigated the pattern of INSTI primary and acquired drug resistance mutations. To this regard, INSTI naïve (n=270) integrase sequences [representing HIV subtypes B (n=92), 01_AE (n=25), C (n=82), A1/A2 (n=22), CRFs (n=21), 02_AG (n=15) and other pure subtypes (n=13)] and INSTI experienced (n=96) individuals from a patient-cohort (collected from Swedish InfCare) were analyzed. Results in INSTI naïve patients indicated the frequency of INSTI primary mutations to be <1% (1/270) and INSTI accessory mutations to be 6.3% (17/270). In addition, the M50I polymorphic mutation was observed in 18% (49/270) of INSTI naïve patients. However, a prevalence of 24% (23/96) primary INSTI mutations was observed in INSTI-experienced patients. The most common mutations observed were N155H, Y143G/R, T66S, E92Q, Q148R, S147G, E138K, G140A/S. Lastly, there was no observable difference between non-B and HIV-1B subtypes in the occurrence of drug-resistant mutations.

Conclusion

No subtype-specific difference was found amongst all INSTI and it was observed that DTG, CAB and BIC had higher antiretroviral potency than RAL and EVG and work well in non-B subtypes. However, follow up research is required to understand the impact of naturally occurring polymorphisms and accessory mutations when they occur with known INSTI mutations

6 DISCUSSION

HIV-1 continues to be a burden in low- and middle-income countries (LMIC) that are mainly infected with non-B HIV-1 subtypes. Regardless of the scaling-up of ARV rollout programs, efforts to contain the pandemic through early detection and virological monitoring of infected patients have not stopped the trend in increasing acquired drug resistance (ADR) (2, 257). The combined results of **paper I, II, III,** make a persuasive argument to support the hypothesis proposed in this thesis that subtype-specific differences impact the outcomes of ARV therapy and that the impact is mainly detrimental to individuals infected with HIV-1 non-B subtypes.

To explain in part the impact on subtype-specific differences, results in **paper I** demonstrated the reduced sensitivity of some HIV-1C PYxE-carrying viruses to PIs when compared to wild type (WT) and HIV-1B viruses. This observation could have multifactorial reasons. For Instance, it could be linked to the mechanism of binding of PIs to HIV-1 protease. To explain further, even though most PIs function by binding in the active site of protease, DRV is an example of an exception. DRV has a unique mechanism of action of binding to protease that is not common for other PIs and this mechanism proposes that DRV can also bind on the surface of one of the enzymes' flexible flaps in the protease dimer (260). Notwithstanding the facts that DRV has a high binding affinity to HIV protease (approximately 100-fold than most PIs) (261), a high genetic barrier to resistance than most PIs (262) and that clinical studies have also shown the superiority of DRV against LPV (263) and ATV (264), there is evidence that DRV can be compromised by single mutations. For example, it has been shown that a single mutation in protease V32I can compromise the antiviral activity of DRV substantially (265). In addition, *in vitro* selection experiments have demonstrated the development of gag mutations that could affect DRV antiviral activity (196). Hence there might be mechanisms of drug resistance that could be specific to either DRV, ATV and LPV. Since viruses used in **paper I,** lacked primary mutations in the protease and were derived from treatment-naïve patients, it may be rational to assume that naturally occurring polymorphisms (NOPs) or mutation(s) in HIV-1C gag could cause phenotypic resistance to PIs in a manner not yet investigated. Furthermore, it can be assumed that potential gag mutation(s) may not show cross-resistance to PI drugs and be specific in acting on the unique mechanisms each PI may have in binding to protease. The observed results of the replication fitness of HIV-1C viruses containing the PYxE motif in gag-p6 region compared to the WT HIV-1C reinforces the potential importance of gag. Other studies have shown similar results (205, 266)

In paper **II**, EFdA was investigated with other NNRTIs and TAF for their *in vitro* potency and subtype-specific response. The broad and subtype-independent activity of EFdA was demonstrated compared to other drugs and EFdA can be recommended as a strong candidate for a long-acting drug that can be administered to HIV-1 patients. Proof of concept for the long-acting drug potential of EFdA has already been confirmed by a recent report (267) that demonstrated a reduction of viral load in animals injected with an implant of EFdA for more than six months. Results from **paper II** give optimism to the future of ARV therapy for non-B subtypes. Considering the challenges HIV-1 infected patients face in adherence to existing clinical drugs (268), the potential of long-act formulations could revolutionize the progress of ARV therapy. However, RPV and ETR results were not encouraging for their use in an HIV-1C dominated setting. To add on, RPV already has a long-acting coformulation with INSTI drug - CAB that has already entered clinical trials (269) and it is worth mentioning that the success of the long-acting formulation in a subtype C dominated setting will require close monitoring of HIV-1C patients. DRMs, even for those known to cause a reduction in fitness of HIV-1 viruses when treated with RPV can be compensated by single mutations that evolve rapidly, leading to the emergence of fit RPV-resistant viruses. For instance, it is already known, that the single mutation E138A/K (which reduces the fitness of RPV-resistant HIV-1C viruses) can be compensated by the development of N348I mutation in the connection domain of RT leading to a E138A/K fit resistant virus (270). Hence regimens containing RPV may not be suitable in an HIV-1C dominated setting.

To conclude, the World Health Organisation (WHO) has recommended the use of INSTIs as part of the regimen for first-line therapy in LMIC (2). Results from **paper III**, suggest that second-generation INSTIs are a better option for non-B subtypes. However, the occurrence of naturally occurring polymorphisms (NOPs), such as M50I, could compromise the efficacy of second-generation INSTIs when they occur with known mutations, such as R263K, as recently shown (271). The observation of NOPs in HIV-1C integrase that have the potential to impact the efficacy of INSTIs was also reported recently for integrase sequences analysed from South Africa (272). However, more mechanistic studies are needed to demonstrate the association of NOPs with major INSTI mutations and evaluate the potency of INSTIs in treating HIV-1 non-B subtypes.

8 CONCLUSION AND RECOMMENDATIONS

The following conclusions and recommendations can be drawn from findings in this thesis:

- Mutations in the gene or changes in the gag-p6 region could significantly limit the options of second-line treatment involving boosted PI in the presence or absence of known protease mutations. More research, involving mechanistic studies is recommended to identify the role of gag mutations or naturally occurring polymorphisms (NOPs) that affect PI susceptibility. Once the mutations are established, genotypic testing assays can be expanded to include the gag gene.

- EFdA is a strong candidate to inhibit non-B subtypes and a good candidate as a long-acting drug to overcome problems associated with adherence that can hamper the success of ARV therapy and lead to the emergence of drug resistance. Furthermore, it is recommended that the use of RPV-based regimens in an HIV-1C dominated setting should be closely monitored and possibly avoided in patients who are in high-risk groups, such intravenous drug users (IDUs), men who have sex with other men (MSM) and sex workers, where there is increased risk of transmitting resistant viruses.

- Finally, it can be concluded that second-generation INSTIs have a high inhibitory potential than first-generation INSTI drugs and, more specifically, in non-B subtypes (such as A-like viruses). However, it is recommended that routine monitoring and adherence support be given to INSTI HIV-1 patients for the surveillance of NOPs that could synergize with know INSTI mutations and lead to the emergence of drug resistance.

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