Cellular and virological mechanisms of HIV-1 dolutegravir-specific resistance

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“If I have seen further than others, it is by standing upon the shoulders of giants.”

- Isaac Newton

Dedication

This thesis is dedicated to:
Dr Mark Arnold Wainberg
(1945 – 2017)
A loving father, caring husband, passionate researcher, fierce HIV/AIDS advocate, devoted mentor, and a Lion among men.

Abstract

Integrase strand transfer inhibitors (INSTIs), the newest class of antiretroviral drugs to be approved for the treatment of HIV-infected individuals, act by inhibiting the essential HIV protein integrase (IN) from inserting the viral DNA genome into the host cell's chromatin. Three drugs of this class are currently approved for use in HIV-positive individuals: raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG). The former two compounds have been reasonably successful in clinical settings but have relatively low genetic barriers to resistance. Furthermore, they share a high degree of cross resistance, which necessitated the development of so-called second-generation drugs of this class that could retain activity against these resistant variants. DTG was approved in 2013, is able to suppress the vast majority of RAL/EVG resistant viruses, and selects for the novel R263K resistance substitution both in tissue culture selection studies and in patients during rare cases of virological failure with this inhibitor.

These antiretrovirals are competitive inhibitors that bind at the enzyme's active site, and so the majority of resistance-associated changes involve a distortion of the geometry of the catalytic core domain of IN and interference with inhibitor binding. The R263K substitution, however, is located within the C-terminus of IN and provides resistance through an unknown mechanism. As this region of the enzyme is acetylated and this post-translational modification is integral to productive infection, Chapter 2 explores the role of acetylation in R263K-mediated DTG resistance. It was found that IN bearing R263K interacted with higher affinity with KAP1, a component of the histone deacetylase I complex, than did wild-type (WT) enzyme. This resulted in protection from histone acetyl-transferase (HAT) inhibitor-mediated DTG sensitization as compared to

WT and RAL-resistant viruses. Inhibition of deacetylation decreased replicative capacity of WT virus while treatment with HAT inhibitors augmented the replication of $HIV_{R263K,}$, suggesting a role for acetylation in regard to both the resistance and fitness phenotypes of the mutated virus.

With the knowledge that the R263K DTG resistance pathway was independent of classical mechanisms of RAL/EVG resistance, Chapter 3 explores the possibility of combining first- and second-generation INSTI resistance mechanisms together in a single protein and virus in order to evaluate the outcomes on IN enzymatic activity, viral infectivity, and INSTI resistance. The combination of R263K with the most common RAL/EVG resistance substitutions resulted in additive, negative effects on IN activity that were mostly not compensated by increased DTG resistance in cell-free assays. In tissue culture, N155H with R263K was the sole combination that resulted in improved infectivity and decreased susceptibility to DTG compared to R263K alone. This warranted further investigation of pathways leading to high levels of DTG resistance.

In Chapter 4, the possibility of selecting a hyper DTG resistant HIV variant using the N155H and R263K resistance pathways is reported. As the N155H substitution is often found in combination with one secondary resistance mutation in patients failing therapy after treatment with RAL or EVG, each of the five most common N155H-associated secondary changes was added into the N155H/R263K background to ascertain IN biochemical activity, viral infectivity, and INSTI susceptibility. Although some of these tertiary changes improved the activity of IN in cell-free assays, all the triply mutated viruses displayed decreased infectivity without any increase in DTG resistance.

Thus, it was concluded that the combination of the N155H and R263K resistance pathways would most likely not be clinically significant.

In Chapter 5, the combination of another RAL- and EVG-associated resistance pathway with R263K was explored, again in an attempt to engineer a fit, hyper-resistant virus. The E157Q change has long been associated with INSTI resistance, but is also a natural polymorphism present in up to 10% of the circulating virus. It was also the only reported change after DTG failure in a highly treatment-experienced patient who also experienced treatment failure with RAL before switching to DTG. Here, it was found that the E157Q substitution had little effect on its own in biochemical assays on IN activity, but was able to partially compensate for the deficits observed with R263K. In tissue culture, the E157Q single mutant displayed wild-type levels of infectivity and hyper-sensitivity to DTG. However, in combination with R263K the polymorphism completely restored the defect in infectivity of the former, while increasing DTG resistance by 10-fold. Thus, the generation of a fit, DTG-resistant HIV-1 variant was shown possible in a laboratory setting.

This thesis characterizes the clinically significant R263K resistance pathway for DTG. It establishes a role of the regulation of cellular acetylation in the susceptibility of HIV to this INSTI, and shows the distinctness of R263K versus classical pathways of resistance towards first-generation INSTIs. As the use of DTG to treat both naïve and experienced patients increases worldwide, the importance of understanding how and why resistance develops in response to this compound cannot be understated. Only through enhancing our knowledge of mechanisms of HIV-1 drug resistance will clinicians be able to design better treatments for their patients and chemists be able to engineer more potent

antiretroviral drugs. This will be essential in reaching the UNAIDS goal of 90-90-90 and bringing the HIV pandemic under control.

Abrégé

Les inhibiteurs d'intégrase (INIs) sont la classe de médicaments antirétroviraux approuvée le plus récemment. Ils agissent en inhibant l'intégrase (IN), l'enzyme essentielle du VIH qui sert à faciliter l'insertion du génome viral dans la chromatine des cellules cibles. Trois médicaments de cette classe sont disponibles pour le traitement des personnes séropositives: raltégravir (RAL), elvitégravir (EVG) et dolutégravir (DTG). Les deux premiers INIs sont relativement efficaces en clinique mais ils ont des barrières génétiques relativement faibles contre la résistance. Aussi, ils partagent un haut degré de résistance croisée entre eux, ce qui a nécessité le développement d'INIs de deuxième génération qui pourraient conserver l'activité contre les virus résistants. DTG a été approuvé en 2013, est capable de supprimer la grande majorité des virus résistants RAL/EVG, et sélectionne la nouvelle mutation de résistance R263K à la fois en culture de tissus et chez les patients lors des rares cas d'échec virologique avec cet inhibiteur.

Ces antirétroviraux sont des inhibiteurs compétitifs qui se lient au site actif de l'enzyme et donc la majorité des changements associés à la résistance aux INIs causent une distorsion de la géométrie du domaine catalytique de l'IN et une interférence avec la liaison des inhibiteurs. La substitution R263K, cependant, est située dans le domaine C-terminal de l'IN et confère la résistance par un mécanisme inconnu. Comme cette région de l'enzyme est acétylée et cette modification post-traductionnelle est intégrale pour l'infection productive, dans le Chapitre 2, le rôle de l'acétylation dans la résistance au DTG procurée par R263K est exploré. Il a été trouvé que la protéine IN portant cette mutation signature de DTG interagit avec une affinité plus élevée avec Kap1, un composant du histone deacetylase complex I. Cela se traduit par le fait que R263K n'est

pas sensible à l'augmentation de la susceptibilité du virus à l'inhibition par le DTG quand les inhibiteurs des histone acetyl-transferases (HATs) sont utilisés avec le virus WT ou un virus résistant au RAL. Les virus WT et R263K sont affectés de manière opposée par l'inhibition de l'acétylation et de la désacétylation dans des cellules productrices de virus, tandis que l'inhibition de la désacétylation diminue la capacité réplicative du virus WT, le traitement par des inhibiteurs de HAT augmente la replication du VIH_{R263K}, ce qui suggère un rôle pour l'acétylation dans la résistance au DTG et la survie du virus mutant.

Le Chapitre 3 explore la possibilité de combiner les mécanismes de résistance aux INIs de première et de deuxième générations dans une seule protéine et un seul virus pour évaluer les effets sur l'activité enzymatique de l'IN, l'infectiosité virale et la résistance aux INIs. La combinaison de R263K avec les substitutions de résistance à RAL et EVG les plus communes a eu des effets négatifs additifs sur l'activité de l'IN qui, en grande partie, n'ont pas été compensés par une augmentation de la résistance au DTG dans des essais biochimiques. Dans les essais en culture de tissus, N155H avec R263K était la seule combinaison qui a eu une infectiosité améliorée et une sensibilité diminuée au DTG comparée à R263K en isolation, ce qui justifiait une étude du développement des niveaux de la résistance au DTG dans le contexte de la combinaison de N155H avec R263K.

Dans le Chapitre 4, la possibilité de sélectionner un variant du VIH hyper-résistant au DTG en utilisant les substitutions de résistance N155H et R263K ensemble est rapportée. Comme la substitution de N155H est souvent trouvée en combinaison avec une mutation de résistance secondaire chez les patients qui échouent leur traitement avec RAL ou EVG, les cinq changements secondaires les plus communs associés à N155H ont été ajouté individuellement à la combinaison N155H/R263K pour l'évaluation

de l'activité biochimique de l'IN, ainsi que l'infectiosité virale et la sensibilité aux INIs. Bien que certaines de ces mutations additionnelles aient amélioré l'activité de l'IN dans des essais biochimiques, tous les virus triplement mutés ont montré une diminution de l'infectiosité sans augmentation de la résistance aux DTG comparé au N155H/R263K. Ainsi, on a conclu que la combinaison des substitutions de résistance N155H et R263K ne serait probablement pas cliniquement significative.

Dans le Chapitre 5, nous avons exploré une autre voie de résistance associée à RAL et EVG en combinaison avec R263K, encore une fois dans le but de fabriquer un virus hyper-résistant contre le DTG. La substitution E157Q a longtemps été associée à la résistance aux INSTIs, mais est également un polymorphisme naturel présent dans presque 10% du virus circulant. C'était aussi le seul changement noté après les échecs successifs sous traitement RAL puis DTG d'un patient qui avait reçu de nombreux traitements différents. Nos résultats montrent que la substitution E157Q a peu d'effet par elle-même sur l'activité de l'IN dans les analyses biochimiques, mais peut compenser partiellement les défauts observés avec la mutation R263K uniquement. En culture de tissus, le virus mutant E157Q a montré des niveaux d'infectiosité identiques aux virus de type sauvage, et une hyper-sensibilité à l'inhibition par le DTG. Cependant, en combinaison avec R263K, E157Q restaure complètement la réplication virale, tout en augmentant 10 fois la résistance au DTG. Ainsi, la génération d'un variant du VIH-1 résistant au DTG a été possible en laboratoire.

Cette thèse caractérise en détail la mutation de résistance R263K contre le DTG. J'ai établi le rôle de la régulation de l'acétylation cellulaire dans la susceptibilité du VIH à cet INI, ainsi que l'indépendance du R263K et des voies classiques de résistance aux

INIs de première génération. Comme l'utilisation de DTG pour traiter à la fois des patients naïfs et expérimentés augmente chaque jour dans le monde entier, l'importance de comprendre comment et pourquoi la résistance se développe contre ce médicament ne peut pas être sous-estimée. Ce n'est qu'en améliorant nos connaissances sur les mécanismes de la résistance du VIH que les cliniciens pourront concevoir les meilleurs traitements pour leurs patients et que les chimistes seront capables de créer des médicaments antirétroviraux plus puissants. Il s'agit de deux étapes essentielles pour atteindre l'objectif de l'ONUSIDA de 90-90-90 et de contrôler la pandémie du VIH.

Preface

This thesis conforms to McGill University Graduate and Post-Doctoral Studies' guidelines for thesis preparation and has been formatted as a manuscript- or article-based thesis. The thesis author is the first author on all co-authored articles in the thesis and has obtained permission from co-authors, where necessary, to include these manuscripts herein. These works will not be used in any other thesis. The following manuscripts are included in the thesis:

Chapter 1:

Anstett, K., Mesplede, T., and M. A. Wainberg (2017). Comparing *the in vitro* and *in vivo* selection of integrase strand transfer inhibitor resistance. *Retrovirology*, submitted.

Distinct contribution to knowledge: This review article summarizes all up-to-date data on the selection of resistance substitutions using integrase strand transfer inhibitors in tissue culture selections and in patients treated with these drugs. It also summarizes the levels of drug resistance measured for various combinations of substitutions in *in vitro* assays. It is meant to serve as a reference guide for those working in the field.

Chapter 2:

Anstett, K., Mesplede, T., and M. A. Wainberg (2017). HIV-1 resistance to dolutegravir is affected by cellular histone acetyl-transferase activity. *Journal of Virology*, submitted.

Original scholarship: This was the first report of the regulation of HIV-1 drug resistance via a post-translational modification. This report established the importance of the acetylation state of both the virus-producing and target cells for proper HIV-1 replication and susceptibility to second-generation INSTIs. It was also the first report showing an increased affinity of HIV integrase bearing an ARV-selected resistance substitution with

a cellular co-factor (KAP1), which helps to explain the altered phenotype of the R263K mutant versus wild-type in response to the inhibition of acetylation. This work creates a new avenue for the investigation of the importance of acetylation in the response of HIV to INSTIs.

Chapter 3:

Anstett, K. et al (2015). HIV-1 dolutegravir-resistance substitution R263K cannot co-exist in combination with many classical integrase inhibitor resistance substitutions. Journal of Virology DOI: 10.1128/JVI.03485-14.

Original scholarship: This manuscript was among the first to show the significance of the N155H pathway in the development of dolutegravir resistance. It was also the first study to combine the DTG-specific resistance mutation R263K with the most common RAL/EVG primary mutations and showed that these pathways were incompatible. This helped to explain the lack of selection of R263K in patients harbouring resistance to first-generation INSTIs. R263K + each of E92Q, Y143R, Q148R, or N155H had never been examined in biochemical or tissue culture-based assays prior to this study.

Chapter 4:

Anstett, K. et al (2015). Dolutegravir-selected HIV-1 containing the N155H/R263K resistance substitutions does not acquire additional compensatory mutations under drug pressure that lead to higher level resistance and increased replicative capacity. Journal of Virology DOI: 10.1128/JVI.01725-15.

Original scholarship: This was the first report of the effects of combining the N155H and R263K resistance pathways on the activity of the HIV integrase enzyme, on viral infectivity, and on resistance to INSTIs. None of these triply mutated viruses had ever

been characterized in either cell-free assays or in tissue culture prior to this report. This study established that although both the N155H and R263K pathways may contribute to DTG failures in the clinic individually, the combination of these pathways was unlikely to be biologically relevant. In keeping with this, these mutations have yet to be observed together in a patient failing treatment with DTG.

Chapter 5:

Anstett, K. et al (2016). Polymorphic substitution E157Q in HIV-1 integrase increases R263K-mediated dolutegravir resistance and decreases DNA binding activity. Journal of Antimicrobial Chemotherapy DOI: 10.1093/jac/dkw109.

Original scholarship: This study was the first to show that an additional amino acid change in HIV-1 integrase could compensate for the fitness defect of the R263K substitution and significantly increase levels of dolutegravir resistance *in vitro*. Previous to this report, the E157Q mutant had never been characterized in terms of DNA binding activity and the E157Q/R263K double mutant had never been characterized at all. In this study we created a fit, highly DTG-resistant HIV-1 variant. This work has clinical relevance given that position 157 in IN is polymorphic and may be present at treatment initiation.

The following are co-authorships not included in the thesis:

Liang, J. et al (2015). The Combination of the R263K and T66I Resistance Substitutions in HIV-1 Integrase Is Incompatible with High-Level Viral Replication and the Development of High-Level Drug Resistance. Journal of Virology DOI: 10.1128/JVI.01881-15.

Cutillas, V. et al (2015). The R262K substitution combined with H51Y in HIV-1 subtype B integrase confers low-level resistance against dolutegravir. Antimicrobial Agents and Chemotherapy DOI: 10.1128/AAC.04274-14.

Wainberg M, et al (2014). The R263K mutation in HIV integrase that is selected by dolutegravir may actually prevent clinically relevant resistance to this compound. Journal of the International AIDS Society DOI: 10.7448/IAS.17.4.19518.

Mesplede, T. et al (2014). Addition of E138K to R263K in HIV integrase increases resistance to dolutegravir, but fails to restore activity of the HIV integrase enzyme and viral replication capacity. Journal of Antimicrobial Chemotherapy DOI: 10.1093/jac/dku199.

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The last three and a half years have passed in a whirlwind. First and foremost, I must thank my supervisor Dr Mark A Wainberg. For you to treat me as anything approaching an equal has been the greatest honour. You've pushed me both academically and politically, always with kindness and respect. You've introduced me to Nobel Prize laureates and federal cabinet ministers, and given me the opportunity to travel around the world. Your unwavering confidence in my abilities is one of the most important factors in the completion of this thesis. I would also just like to say that *if* I have become a capitalist it is entirely your fault. Your sudden passing has shaken everyone who knew you to their cores. You were an inspiration and proof that with hard work, passion, and tenacity no dream is impossible. We love you dearly, and we miss you already.

To Dr Thibault Mesplede, I sincerely could not have done this without you. It is very rare to find someone who understands how I think as instinctively as you do. It is even rarer to find someone who from the very beginning had as much faith in my intelligence and abilities as you did. I cannot thank you enough for the opportunities you've given me, for the help and support you've provided, and for being my friend and confidant for the last three years.

For the lab members who came before me, I do truly stand on the shoulders of giants. To Drs Peter K Quashie and Yingshan Han, thank you for perfecting our biochemical assays; I would not be completing my thesis in four years without you. Likewise, thank you to Dr Diane Singhroy for all your advice, both technical and personal. Dr Kramer, your advice and guidance have been very insightful, especially now as I try to follow in your footsteps. To my thesis-writing, soon-to-be-Dr, partner-in-crime Said

Hassounah, thank you for never getting too annoyed with me, and for your genuine interest in my research projects.

To the current crew of students at the McGill AIDS Centre, you guys have been so kind, supportive, and fun. I know you'll go on to do big things. Nathan, I love you, and you'll just have to deal with it. Hanh, thank you for letting me steal all your reagents and your office, and for keeping me in constant supply of candy and cookies. Maureen and Illinca, you've been the best friends a gal could ask for. There were some days when the only thing I had to look forward to was getting coffee with you so thank you for always being there. Estrella, Cesar, Sue- thank you for always doing your best to help me with whatever emergency I had that day. Dr Bluma Brenner, for you to say that I remind you of yourself is the nicest thing anyone has ever said to me. I wish that I could find anything to be as passionate about in my life as you continue to be about your work. Truly, you inspire me everyday. To my students: Marion, Rob, and even Adam (jkjk), you were the best students I could have ever asked for. You had to put up with my scatter-brain and my inability to do basic math, and you all did so beautifully. I know you'll all go on to be amazingly successful scientists.

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are the strongest person I know and just to have you in my life and on my side has been an amazing buoy these last… 15 years?! Je voulais aussi dire merci à Sibok et toute ma famille Patenaude- vous m'avez sauver la vie.

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To the Newmans and the Marshalls, you are two larger-than-life families. Thank you for welcoming me into your midst and for the care that you've shown me. And thank you for your son, Sean. The single, greatest thing in my life. You have been the light in all the darkness, the single, shining, true thing that led me through *everything*. You are my best friend, the love of my life, and the one thing without which none of this would have happened. My love for you and the gratitude I feel cannot be put into words. But, believe you me, I will spend the rest of my life trying.

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List of Abbreviations

95% CI – 95% confidence interval

3TC - Lamivudine

A – Adenine

AA – Anacardic acid

ABC - Abacavir

AIDS – Acquired immunodeficiency syndrome

ANOVA – Analysis of variance

APOBEC3G – Apolipoprotein editing complex catalytic subunit 3G

APV - Amprenavir

ARV – Antiretroviral drug

ATV - Atazanavir

AZT - Zidovudine

BAF – Barrier to autointegration

BIC - Bictegravir

BST-2 – Tetherin

c - Cobicistat

CD4 – Cluster of differentiation 4

CA – Capsid protein

CBMCs – Cord blood mononuclear cells

CC50 – 50% cytotoxic concentration

CCD – Catalytic core domain

CCR5 – CC-chemokine receptor 5

cDNA – Complementary DNA

CNRS – Centre national de la recherche scientifique (French ministry of Education and Research)

Co-IP – Co-immunoprecipitation

CPM – Counts per minute

cpz – Chimpanzee

CRF – Circulating recombinant form

CRM1 – Chromosomal maintenance 1

CTD – C-terminal domain

CTG - Cabotegravir

CXCR4 – CXC-chemokine receptor 4

D – Aspartic acid

d4T - Stavudine

DC – Dendritic cell

ddI – Didanosine

DLV – Delavirdine

DMEM – Dulbecco Modified Eagle medium

DNA – Deoxyribonucleic acid

dNTP – deoxyribonucleotide

DRV - Darunavir

DTG – Dolutegravir

DTT – Dithiothreitol

E – Glutamic acid

EC50 – 50% effective concentration

EDTA – Ethylenediaminetetraacetic acid

EFV - Efavirenz

Env – Envelope glycoprotein

ETR - Etravirine

EVG – Elvitegravir

FC – Fold change

FDA – United States Food and Drug Administration

FOS-APV - Fosamprenavir

FTC - Emtricitabine

G – Guanine

Gag – Group specific antigen

GFP – Green fluorescent protein

GFP-INB – GFP-tagged integrase protein

Glu – Glutamic acid

gor – Gorilla

HAART – Highly active antiretroviral therapy

HAT – Histone acetyl-transferase

HATi – Histone acetyl-transferase inhibition

HDAC – Histone deacetylase

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HHCC – Histidine-Histidine-Cysteine-Cysteine

HIV – Human immunodeficiency virus

hpi – hours post-infection

HRP – horseradish peroxidase

IC50 – 50% inhibitory concentration

IDV – Indinavir

IL - Interleukin

IN – Integrase enzyme

INB – Integrase enzyme from HIV-1 subtype B

INI – Integrase inhibitor

INI1 – Integrase interactor 1

INSTI – Integrase strand transfer inhibitor

JAK – Janus kinase

K – Lysine

KAP1 – KRAB-associated protein 1

LEDGF/p75 – Lens epithelium-derived growth factor

LPV – Lopinavir

LRA – Latency reversing agent

LTR – Long terminal repeat

M – HIV-1 main group

MA – Matrix protein

MHC – Major histocompatibility complex

Mg – Magnesium

Mn – Manganese

mTOR – Mammalian target of rapamycin

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MVC – Maraviroc

MxB – Myxovirus resistance B protein

N – HIV-1 non-M/non-O group

NA – No inhibitor control

NC – Nucleocapsid protein

Nef – Negative regulatory factor

NFV - Nelfinavir

NHP – Non-human primate

NIH – National Institutes of Health

NRTI – Nucleoside reverse transcriptase inhibitor

NNRTI – Non-nucleoside reverse transcriptase inhibitor

NTD – N-terminal domain

Nup – Nucleoporin

NVP - Nevirapine

O – HIV-1 outlier group

PBS – Primer binding site or phosphate buffered saline (context-dependent)

PD-1 – Programmed-death receptor 1

PI – Protease inhibitor

PIC – Pre-integration complex

ppt – Polypurine tract

PR – Protease enzyme

PRMT6 – Protein arginine methyl-transferase 6

PVDF – Polyvinylidene fluoride

R – Arginine

RAL - Raltegravir

RANBP2 – RNA binding protein 2

Rev – Regulator of the expression of viral proteins

RhoR - Rhodamine

rIC50 – relative 50% inhibitory concentration

RLU – Relative luciferase units

RNA – Ribonucleic acid

RNaseH – Ribonuclease H

RPV - Rilpivirine

RRE – Rev response element

RT – Reverse transcriptase enzyme

RTV/r – Ritonavir

SAHA - Vorinostat

SAMHD1 - SAM domain and HD domain-containing protein 1

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Standard error of the mean

SERINC – Serine incorporator

SIV – Simian immunodeficiency virus

smm – Sooty manabey monkey

SQV - Saquinavir

SU – Surface protein (Gp120)

SUMO – Small ubiquitin-like modifier

SWI/SNF – Switch/sucrose non-fermentable

T-20 – Enfuvirtide

TAM – Thymidine analog mutation

Tat – Transactivator protein

TAR – Transactivating response element

TCA – Trichloroacetic acid

TDF – Tenofovir

TLR – Toll-like receptor

TM – Transmembrane or fusion protein (Gp41)

TPV - Tipranavir

TRIM5α – Tripartite motif containing protein 5 alpha

TRIM28 – Tripartite motif containing protein 28

tRNALys3 – Lysine transfer RNA 3

UNAIDS – The Joint United Nations Programme on HIV/AIDS

vDNA – Viral DNA

Vif – Viral infectivity factor

Vpr – Viral protein R

Vpu – Viral protein U

Vpx – Viral protein X

WT – wild-type

Chapter 1
Introduction

1.1 Origins and Epidemiology of Human Immunodeficiency Virus Infection

In 1981, acquired immunodeficiency syndrome (AIDS) was recognized as a new disease which afflicted many men who have sex with men in large US cities and rendered them particularly sensitive to opportunistic infections and malignancies (1, 2). Doctors noted that an increasing number of individuals from this population began presenting with low CD4 T cell numbers. However, there did not seem to be a common cause for the increased immunodeficiency (3, 4). This was the beginning of one of the deadliest infectious pandemics in recent history.

Two years of intense research and public attention led to the discovery of the retrovirus now known as human immunodeficiency virus I (HIV-I) that was present in the cells of a swollen lymph node from an AIDS patient in 1983.In the following year, this virus would be shown to be the causative agent of the disease (5-7). Françoise Barré-Sinoussi and Luc Montagnier, shared the 2008 Nobel Prize in Physiology or Medicine (along with Harald zur Hausen for his work on oncogenic human papilloma virus infection) for their central roles in this discovery. However the work of Dr Robert Gallo and colleagues was also instrumental in these early characterizations (8). To date, over 35 millions people worldwide have succumbed to AIDS-related illnesses and there are currently more than 36 million people living with HIV, the vast majority of whom live in Sub-Saharan Africa (Figure 1.1) (9).

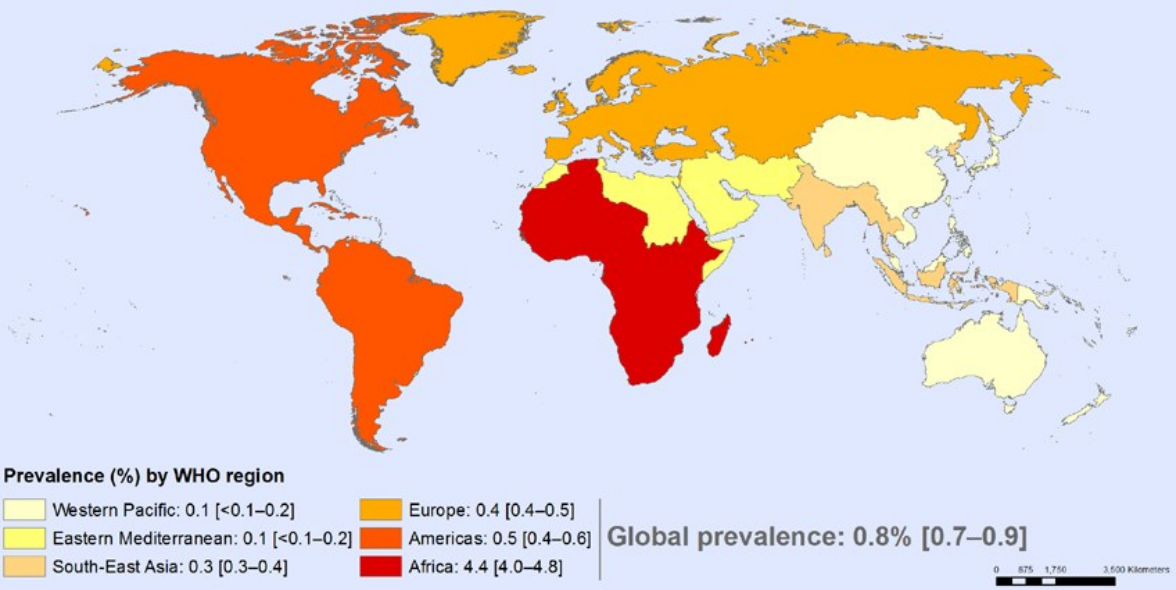


Figure 1.1: Global distribution of HIV-1 infection among adults aged 15-49 years in 2015 (adapted from (9)).

We now know that HIV-1 and HIV-2 are the causative agents of AIDS. Whereas HIV-2 is geographically limited to western Africa and less likely to progress to AIDS in infected individuals, HIV-1 has diversified into many subgroups and spread across the globe (10, 11). HIV-1 is subdivided into groups M (main), O (outlier), N (non-M/non-O), and recently P (restricted to a small group of patients from Cameroon); each of the groups is believed to be the result of an independent cross-species transmission episode from non-human primates (NHPs) (12-15). M is the group responsible for the majority of the global pandemic and is further subdivided into subtypes A through D, F through H, J and K (Figure 1.2); circulating recombinant forms (CRFs) contain genomic material from more than one subtype and are thought to arise when an individual is infected with more than one subtype of virus (16). B is the most common subtype in the Americas, Western Europe, Australia, and China whereas Subtype C predominates in Southern and Eastern Africa and India (17).

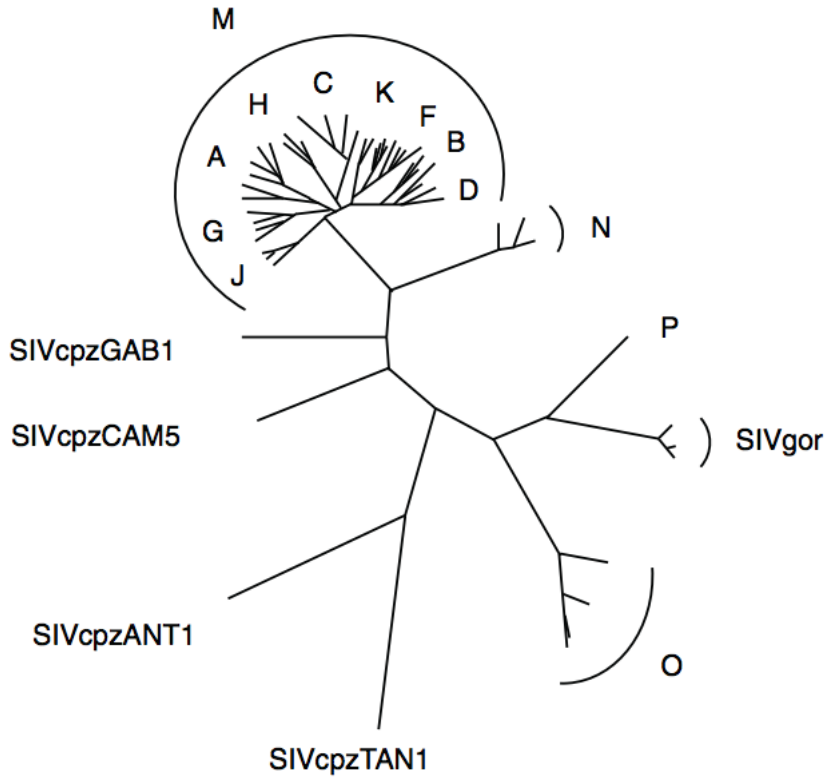
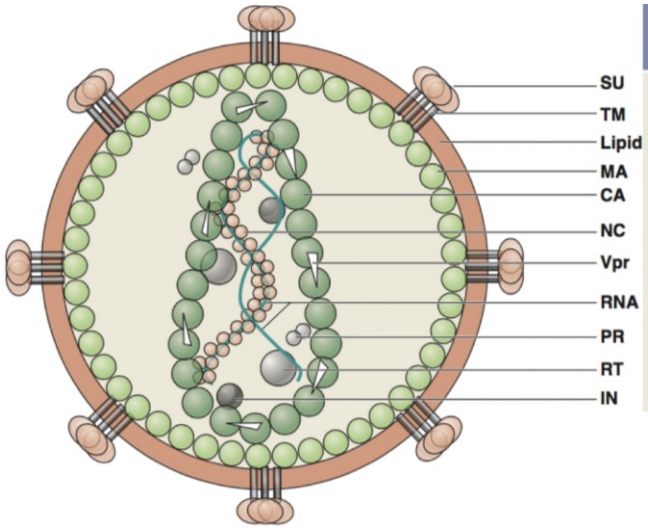


Figure 1.2: Phylogenetic relationships between different HIV-1 groups, subtypes, and the closely related SIV strains (adapted from (18)). “cpz” denotes chimpanzee origin, “gor” gorilla, and “GAB1”, “CAM5”, “ANT1”, and “TAN1” specific locations in Africa.

As mentioned above, the current groups of HIV arose from multiple instances of cross-species transmission. Many species of NHPs harbour simian immunodeficiency viruses (SIVs) that are thought to have crossed the species barrier into humans through cutaneous contact of bodily fluids during traditional bush meat hunting activities in Africa (19, 20). SIV infection in sooty mangabeys and African green monkeys appears to be asymptomatic, while there are some AIDS-like pathologies in SIV-infected chimpanzees (20, 21). HIV-2 is most closely related to the SIV that circulates in sooty mangabeys and thus it has been inferred that the virus passed to humans from this NHP (22). For the same reason HIV-1 groups M and N are thought to be of chimpanzee origin; O and P may also derive from chimpanzees but transmission from the closely related strain of gorilla

SIV cannot be ruled out (3). As the driver of the global pandemic, we will focus specifically on HIV-1 group M in this thesis.

HIV is a retrovirus of the *Lentivirus* genus of the *Retroviridae* family. It is an enveloped virus approximately 100 nm in diameter studded with viral envelope (Env) proteins gp120 and gp41. Under the cell-derived lipid bilayer lies the matrix (MA) protein while the capsid (CA) protein defines the dense viral core within which the viral RNA genome is covered by nucleocapsid (NC) and where reverse transcriptase (RT), integrase (IN), protease (PR), Vpu, Vif, Vpr, Nef, and various cellular factors are also found (Figure 1.3) (23). This family of viruses is characterized by its ability to integrate its genome into the host cell and persist in a latent state for an indefinite amount of time. These viruses also tend to be highly genetically diverse due to the high error rate and lack of proofreading capacity of the RT enzyme, which leads to multiple, slightly differentiated viruses (known as a "quasispecies") circulating in an infected individual (24, 25).



Name	Abbreviation	Alternative name (M. Wt. in KDa)
Matrix	MA	p17
Capsid	CA	p24
Nucleocapsid	NC	p7
Protease	PR	p14
Reverse transcriptase	RT	p66/51
Integrase	IN	p32
Surface protein	SU	gp120
Transmembrane protein	TM	gp41
Virion protein R	Vpr	p15

Figure 1.3: Diagram of a HIV virion (left) and list of HIV structural proteins (right) (adapted from (23)).

1.2 HIV-1 life cycle

HIV infection is predominantly transmitted through mucosal sites during sexual intercourse; however, intravenous drug use and mother-to-child transmission do also occur (26, 27). Owing to CD4 and CXC-chemokine receptor 4 (CXCR4)/CC-chemokine receptor 5 (CCR5) receptor/co-receptor usage, HIV is able to infect T cells, macrophages, monocytes, and dendritic cells (DCs) (23). Early in infection, CCR5-using viruses (termed R5-tropic) predominate and a switch to X4-tropism is usually a hallmark of the progression towards AIDS in infected individuals (26).

During the course of sexual infection, HIV encounters the epithelial cells of the genital tract and infects mucosal CD4+ T cells and DCs, which then migrate to draining lymph nodes where a plethora of target cells are found (28). This is how systemic HIV infection is established. It takes approximately 24 hours for HIV to complete its replication cycle and produce infectious progeny virions (29). The steps in the HIV life cycle are detailed below and in Figure 1.4.

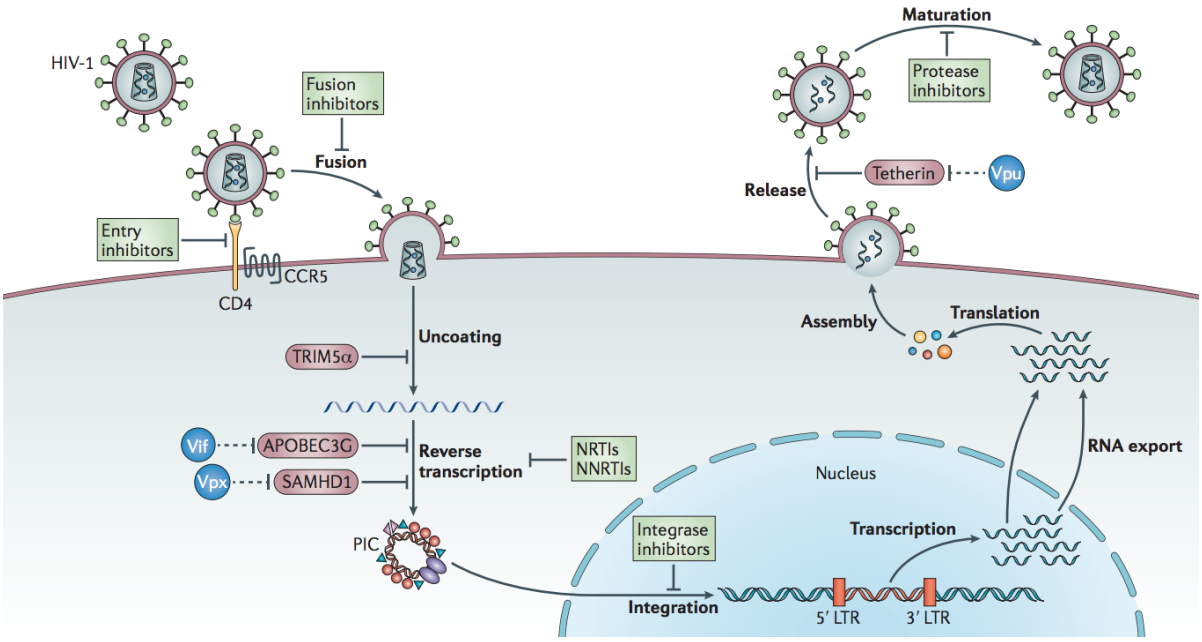


Figure 1.4: Overview of the HIV-1 replication cycle (adapted from (30)).

1.2.1 Entry

The outer layer of the HIV virion is studded with trimers of non-covalently bound Env glycoproteins Gp120 and Gp41 (see Figure 1.3). Gp120 binds to the CD4 receptor on the surface of susceptible cells which triggers a conformational change allowing the viral protein to engage with either the CXCR4 or CCR5 co-receptor (in rare cases alternate receptors are also used) (31, 32). This dual cellular attachment brings the viral and cellular membranes into closer proximity, allowing for the insertion of the fusion peptide Gp41 and the release of the viral core into the cytoplasm (33).

1.2.2 Reverse transcription

There is some debate as to whether CA uncoating or reverse transcription of viral RNA occurs first upon core entry and these processes are likely coupled (34). The host restriction factor tripartite motif containing protein 5 alpha (TRIM5α) targets CA to block retroviral replication. However, HIV has evolved to evade human TRIM5α (35). Host

protein myxovirus resistance B (MxB) has also been recognized as a restriction factor for its interaction with CA (36).

The RT enzyme converts the 9.2 kb single stranded RNA genome into double stranded DNA as follows (see also Figure 1.5): $tRNA^{Lys3}$ binds to the primer binding site (PBS) in the 5' long terminal repeat (LTR) of the HIV plus strand RNA and serves as a primer for RT to initiate cDNA synthesis (37). As DNA is synthesized, the RNA template is degraded through the concomitant action of the RNaseH domain of RT, leaving the minus strand strong-stop DNA which then hybridizes to the 3' end of the RNA template through the interaction of the complementary R terminal repeats (38). DNA synthesis again proceeds and all of the RNA template is degraded save for the polypurine tract (PPT), which then serves as a primer for plus-strand DNA synthesis. After a second strand transfer event the $tRNA^{Lys3}$ is degraded and the PBS is also replicated (39).

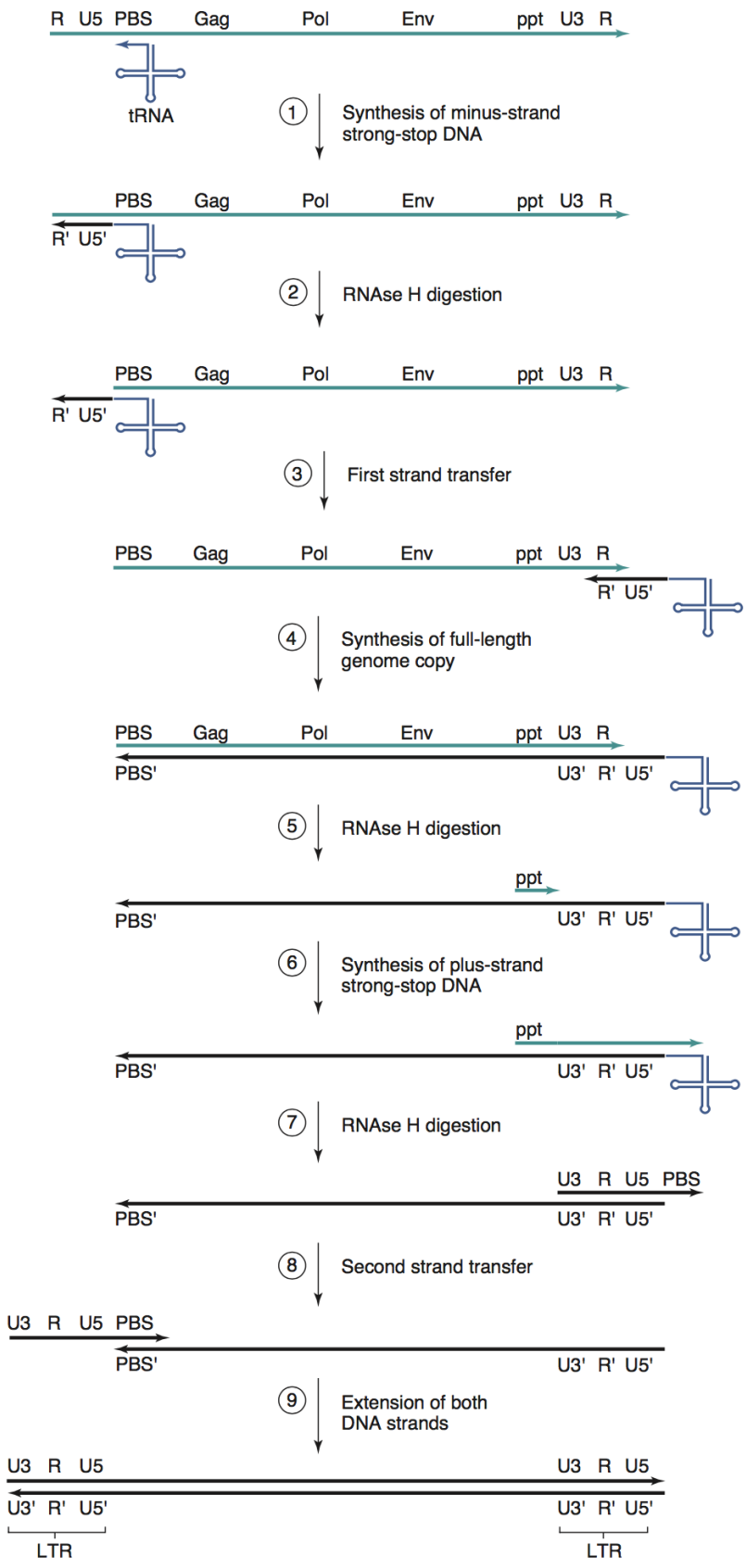

R U5 PBS Gag Pol Env ppt U3 R
tRNA
1
Synthesis of minus-strand strong-stop DNA
PBS Gag Pol Env ppt U3 R
R' U5'
2
RNAse H digestion
PBS Gag Pol Env ppt U3 R
R' U5'
3
First strand transfer
PBS Gag Pol Env ppt U3 R
R' U5'
4
Synthesis of full-length genome copy
PBS Gag Pol Env ppt U3 R
PBS'
U3' R' U5'
5
RNAse H digestion
ppt
PBS'
U3' R' U5'
6
Synthesis of plus-strand strong-stop DNA
ppt
PBS'
U3' R' U5'
7
RNAse H digestion
U3 R U5 PBS
PBS'
U3' R' U5'
8
Second strand transfer
U3 R U5 PBS
PBS'
U3' R' U5'
9
Extension of both DNA strands
U3 R U5
U3 R U5
U3' R' U5'
U3' R' U5'
LTR
LTR

Figure 1.5: Steps in retroviral reverse transcription. Blue lines correspond to RNA, black lines DNA (adapted from (33)).

Apolipoprotein editing complex catalytic subunit 3G (APOBEC3G) is a host restriction factor that can induce hypermutations to the HIV genome during reverse transcription. It is a cytidine deaminase that, when packaged into virions in virus-producing cells, will cause G to A hypermutations by deaminating deoxycytidine to deoxyuridine (40). These mutations are likely to be highly deleterious for the virus, but may also serve to introduce viral escape mutations and further the adaptation of HIV. APOBEC3G is countered by the viral infectivity factor (Vif) which promotes the ubiquitination and subsequently proteosomal degradation of the restriction factor (41).

Another restriction factor that acts to block HIV replication at the reverse transcription step is the SAM domain and HD domain-containing protein 1 (SAMHD1). This nucleotidase is expressed in DCs and other myeloid cell lineages and acts by hydrolizing the cellular dNTP pool to a level below that required by HIV to replicate. SIV from sooty mangabeys (SIV_{smm}) and HIV-2 both encode viral protein x (Vpx) which is absent in HIV-1 and binds to SAMHD1 and signaling for proteosomal degradation (42).

The HIV pre-integration complex (PIC) is composed of the reverse transcribed, double stranded cDNA genome, MA, IN, Vpr, and many cellular co-factors. The PIC travels along the microtubule network to the nuclear pore, where a nuclear localization signal present on one or more viral proteins triggers the entry of the viral components into the host cell's nucleus (43).

1.2.3 Integration

1.2.3.1 Integrase

IN is the only virally encoded protein necessary for the insertion of the viral genome into host cell chromatin. It contains three domains: The N-terminal domain (NTD), the catalytic core domain (CCD), and the C-terminal domain (CTD) (see Figure 1.6) (44). The NTD contains a HHCC motif that co-ordinates a zinc atom, which is involved in stabilizing the native fold of the protein. The NTD also contains residues that have been shown bind DNA and participate in multimerization, as IN is functional as a dimer of dimers (45). The CCD contains the catalytic triad of the IN enzyme, consisting of an Asp-Asp-Glu (DDE) motif which requires the co-ordination of a divalent cation (either Mg^{2+} or Mn^{2+}) for activity. This region also contains several conserved residues that are important for IN-DNA interactions. Finally, the CTD is a less structured and less evolutionarily conserved domain. The CTD has been shown to bind DNA in a non-specific fashion, and plays a role in protein multimerization. While it has been shown in cell-free assays that only the CCD is essential to enzymatic activity, both the NTD and CTD stimulate this activity (IN structure and function is reviewed in (46)).

Figure 1.6: Domains of HIV-1 integrase. NTD is in purple (HHCC motif is shown), CCD is in blue (DDE catalytic triad is shown), and CTD is in red (adapted from (45)).

IN catalyzes the insertion of the viral genome into the host cell's chromosome through two concerted actions: the cleavage of the phosphodiester bond attaching the two terminal 3' nucleotides in both viral LTRs to produce reactive hydroxyl groups, and the nucleophilic attack of these hydroxyl groups on the phosphodiester bond in the host

cell DNA which results in a trans-esterification that joins the two pieces of DNA (47). These two enzymatic functions of IN are referred to as 3' processing, which occurs after cDNA synthesis prior to nuclear translocation, and strand transfer, which occurs in the nucleus (33). The insertion of viral DNA into the host cell genome is completed by the host's own gap repair machinery, which will cleave the unpaired 5' dinucleotides from the LTR and join the resulting DNA ends (48). Figure 1.7 details these processes.

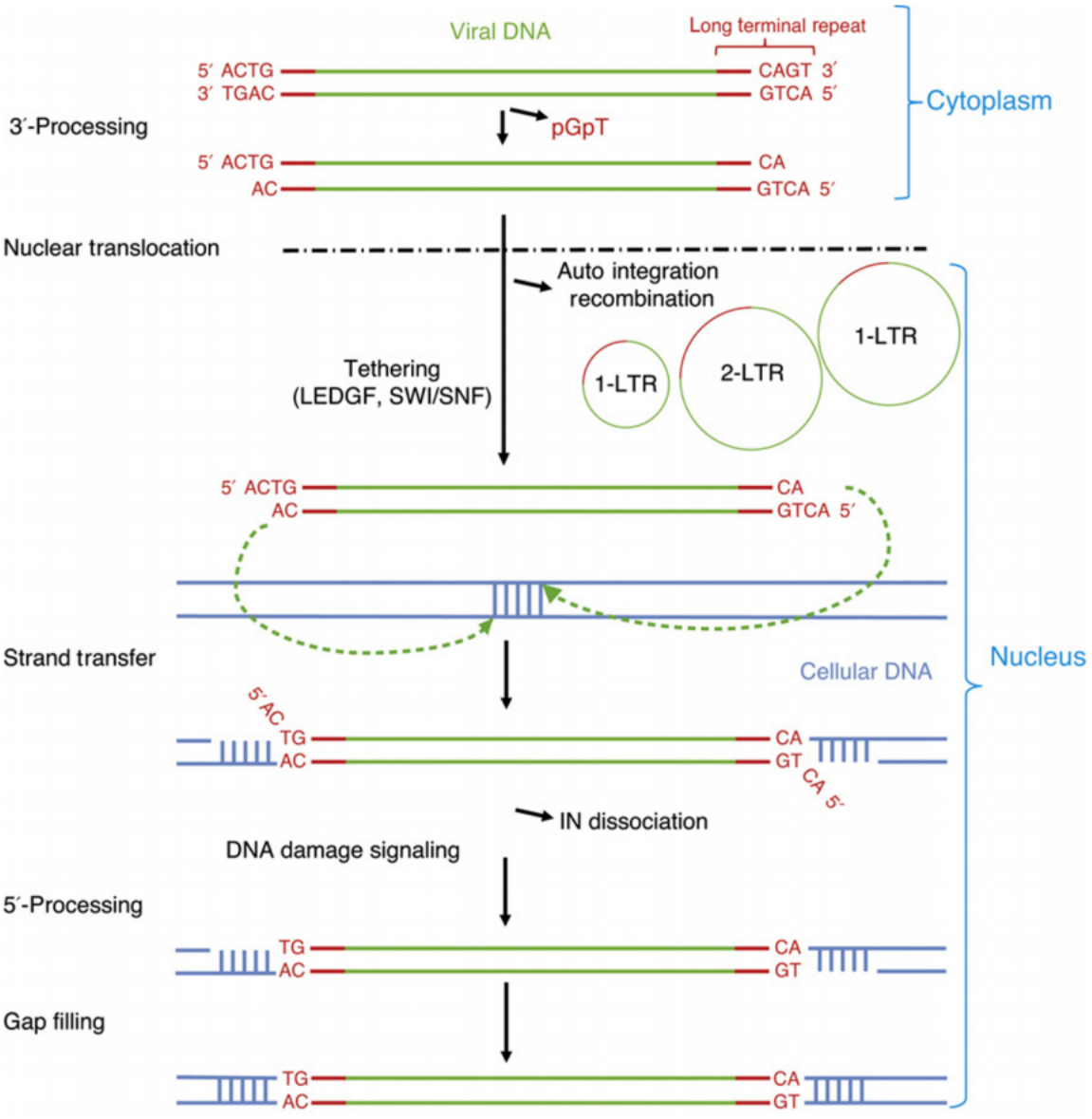


Figure 1.7: Overview of the enzymatic reactions of HIV integrase (adapted from (49)).

There are many cellular co-factors that interact with HIV IN. The first to be discovered was aptly named integrase interactor 1 (INI1), a component of the SWI/SNF complex that is involved in the efficient integration of viral DNA (vDNA) into stable nucleosomes (50). A factor termed barrier to autointegration (BAF) binds to IN and prevents the autointegration of the viral LTR into viral DNA (51), while transportin-3, RANBP2, importin-α, and Nup153 and Nup160 have all been shown to bind to IN and facilitate PIC nuclear import (52-54).

1.2.3.2 LEDGF-IN interaction

The most researched interactor of IN is lens epithelium-derived growth factor (LEDGF/p75). LEDGF co-localizes with IN in the nucleus of infected cells and acts to tether the IN-vDNA complex to specific host genomic loci, areas enriched for open chromatin (48, 55). It is also believed to recruit other cellular factors to the PIC thereby further facilitating integration (56). Knockdown of LEDGF has been shown to severely impact retroviral replication and integration site selection (48). Because of this, disruption of the interaction between LEDGF and IN has become an area of growing research for novel anti-HIV therapeutics. Many groups are actively studying the effects on HIV replication of blocking IN-LEDGF binding by designing small molecules to allosterically block the binding interface between the two proteins (57, 58). It was through investigation of these compounds that the discovery occurred of the importance of the LEDGF in proper integrase multimerization, and that aberrant oligomerization of IN led to the production of non-infectious HIV particles, likely due to altered IN-DNA and IN-RNA interactions within

the virion (59, 60). Subsequent characterizations went on to implicate IN residues 264 and 266 in nucleic acid binding and protein multimerization (61, 62).

1.2.3.3 Post-translational modifications of IN

IN is highly post-translationally modified by host cell proteins. It has been shown to be ubiquitinated, SUMOylated, phosphorylated, and acetylated (reviewed in (63)). These modifications, as well as the key cellular proteins that orchestrate them, are summarized in Table 1.1. For the purposes of this thesis, we will go into more detail regarding the acetylation and deacetylation of HIV IN.

Human proteins	PTM type	Interaction sites in IN	Mechanisms
LEDGF/p75	Ubiquitination	W131, W132, 161–170	Inhibits Ub proteasome degradation
Ku70	Ubiquitination	230–288	Inhibits ubiquitination by decreasing cellular ubiquitin level and deubiquitinates IN through their interaction
hRad18	Ubiquitination	NA	Inhibits ubiquitination
VBP1	Ubiquitination	43–195	Promotes ubiquitination by targeting IN to E3 ligase
Cul2/VHL ligase	Ubiquitination	NA	Acts as Ub E3 ligase and promotes ubiquitination
p300	Acetylation	264–288	Acetylates IN, increases IN affinity to DNA, and promotes integration
GCN5	Acetylation	244–288	Acetylates IN, enhances enzymatic activity of IN
KAP1	Acetylation	NA	Binds and deacetylates IN by recruiting HDAC1, reduces integration
JNK	Phosphorylation	NA	Phosphorylates IN
Pin1	Phosphorylation	NA	Binds phosphorylated IN, leading to conformational changes and stabilization of IN from ubiquitination

Table 1.1: List of cellular factors known to post-translationally modify and/or interact with post-translationally modified HIV integrase (adapted from (63)).

During acetylation, a histone acetyl-transferase enzyme (HAT) will transfer an acetyl group from coenzyme A to the ε-amino group of lysine residues and this covalent modification is reversed by histone deacetylases (HDACs) (64) (Figure 1.8). This process was first described as it pertained to the regulation of chromatin structure, but has since been recognized to affect the activity of many non-histone proteins, including proteins of non-cellular origin (65).

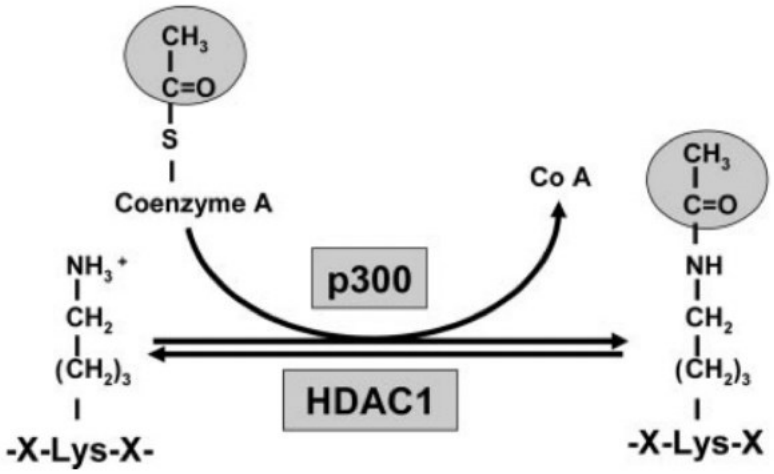


Figure 1.8: Mechanism of acetylation and deacetylation regulated by p300 and HDACI, respectively (adapted from (66)).

The *in vitro* and *in vivo* acetylation of HIV-1 IN at lysines (K) 264, 266, and 273 has been shown to be mediated by both p300 and GCN5, which are cellular HAT enzymes (67, 68). GCN5 has also been shown to acetylate residue K253, but this modification was dispensable for IN catalytic activities and viral infectivity in general. The last 24-44 residues of IN constitute the binding site for both HATs (69). The acetylation of the three residues is essential for IN DNA binding, strand transfer, and viral replication. As with other non-histone DNA binding proteins, the acetylation of IN is thought to increase the affinity of the CTD for DNA (67, 70). Recently, it was also shown that residues 264 and 266 are integral to both IN RNA binding and multimerization, and K264 (along with arginine (R) 263) has been implicated in PIC nuclear translocation (61, 71, 72).

Acetylation can be a dynamic post-translational modification, and HIV-1 IN is also deacetylated at the 3 residues mentioned above by the histone deacetylase I (HDACI) complex *in vitro* and *in vivo.* The complex is recruited through its component

KAP1/TRIM28, which binds directly to IN. In accordance with the results of the acetylation of IN, deacetylation leads to decreased levels of integration (73). Thus, the activity of KAP1 appear to restrict HIV infection.

1.2.4 Genomic transcription and mRNA nuclear export

One of the hallmarks of HIV infection is that, post-integration, the viral genome can reactivate or remain dormant for an indefinite amount of time. There are many factors that promote both the silencing and the reactivation from latency of HIV (reviewed in (74)). Host RNA polymerase II will initiate transcription of the HIV genome from the 5' LTR (75). HIV transactivator protein (Tat) will then bind to the transactivating response element (TAR) located in the 5' LTR to enhance the initiation and rate of transcription (76). Initially, the mRNA that is produced is fully spliced and exported to the cytoplasm in the canonical cellular fashion. This spliced mRNA codes for Tat, regulator of the expression of viral proteins (Rev), and Nef. Rev facilitates the export of non- and partially-spliced HIV RNAs to the cytoplasm through binding to the Rev response element (RRE) present in the transcripts and through engagement with the nuclear export factor chromosomal maintenance 1 (CRM1) (23). See Figure 1.9 for details on HIV RNAs.

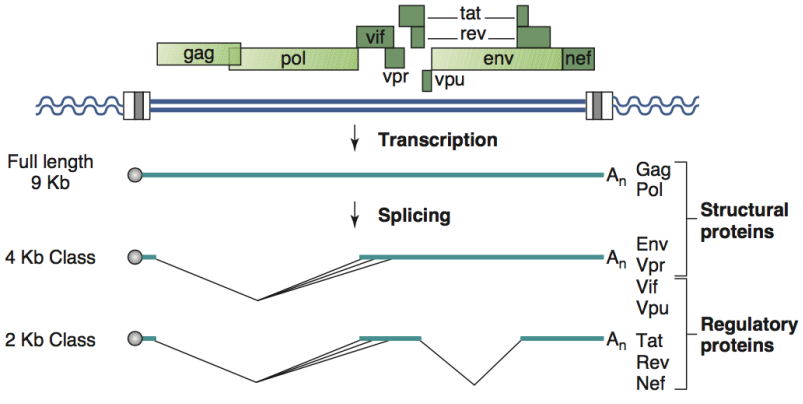


Figure 1.9: Genome organisation and different RNA species of HIV-1 (adapted from (23)). Both the 9Kb and 4Kb class contain the RRE element to facilitate nuclear export via Rev.

Protein arginine methyl-transferase 6 (PRMT6) is also a host restriction factor against HIV infection. PRMT6 methylates Tat, Rev, and NC. Methylation of Tat leads to a decreased function for this protein, thereby also decreasing HIV transcription (77). Methylation of Rev reduces its binding to the RRE, and NC methylation has been shown to decrease the protein's capacity to anneal, which is important for proper functioning (78, 79).

1.2.5 Protein translation and particle formation

Gag and Gag-pol are translated in the cytoplasm. HIV-1 is a C-type retrovirus; particle nucleation initiates at the plasma membrane by virtue of the MA and NC proteins. HIV favours budding from sites of lipid enrichment (80). The Env protein traffics through the endoplasmic reticulum where it is glycosylated and often associated with CD4 (81). Nef and Vpu promote the ubiquitination and proteosomal degradation of CD4 in order to release Env, which is subsequently processed into Gp120 and Gp41 (75). Nef also prevents the detection of the infected cell by the immune system by downregulating the major histocompatibility complexes (MHCs) from the cell surface (82). The main function of NC is to recruit two copies of the viral genome into the budding virion. Immature virions bud from an infected cell, after which the viral PR cleaves Gag-pol to PR, RT, IN, CA, MA, NC, and p6 (Figure 1.10). These cleavages facilitate the formation of the mature, dense viral core and are essential to subsequent infection of new susceptible cells (75).

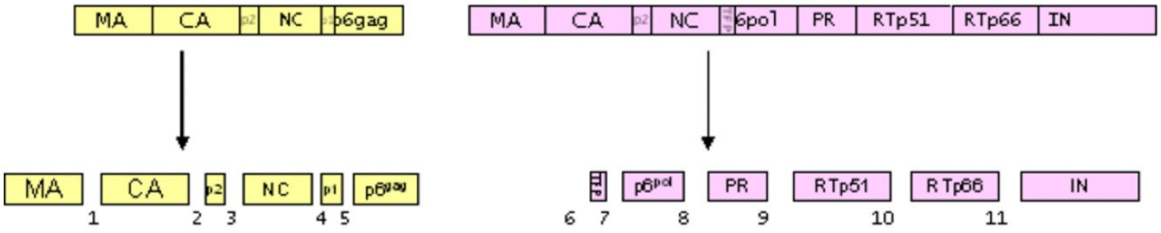


Figure 1.10: Cleavage sites of HIV-1 protease. Left: Gag, right: Gag-pol (adapted from (83)).

The host protein tetherin (also known as BST-2) acts at this stage to block the release of HIV particles from an infected cell by binding to Env, but is antagonized by Vpu (84). Recently, serine incorporators (SERINCs) 3 and 5 have also been implicated in HIV-1 restriction. Although the mechanism of restriction is not completely clear, incorporation of SERINCs into viral particles appears to greatly diminish their infectivity (85, 86). Nef has been shown to downregulate SERINCs from the surface of the plasma membrane, thereby preventing their incorporation into budding virions (87).

1.3 Antiretroviral drugs

Many of the aforementioned steps in the viral life cycle have become targets for antiretroviral drugs (ARVs). There are now approved drugs that target CCR5 binding/entry, RT, IN, and PR. Notably, as there is still no cure for HIV infection, patients are required to take highly active antiretroviral therapy (HAART) for the entirety of their lives. To further complicate matters , antiretroviral drug resistance has been documented for every class of inhibitors that are currently available, highlighting the need for continued research into the mechanisms of HIV drug resistance and the advent of new therapies (88). Due to the error-prone nature of the HIV RT enzyme (discussed above), as many as 3 X 10^5 new mutations are generated in the HIV genome per replication cycle,

producing a quasispecies of HIV variants (25). If one of these species has a mutation that provides a selective advantage for replication in the presence of ARVs it will out-compete other viral forms to become the dominant species (89). This is why the drug classes detailed below are nearly always administered as cocktails of two or more drugs in HAART. Table 1.2 summarizes all the currently available ARVs for treatment of HIV infection.

Drug class	Brand name	Generic name	Manufacturer
Entry inhibitors	Selzentry	Maraviroc (MVC)	Pfizer
	Fuzeon	Enfuvirtide (T-20)	Hoffmann-La Roche, Trimeris
Pharmokinetic enhancers	Norvir	Ritonavir (RTV/r)	Abbott Laboratories
	Tybost	Cobicistat (c)	Gilead Sciences
Integrase inhibitors	Isentress	Raltegravir (RAL)	Merck & Co
	Vitekta	Elvitegravir (EVG)	Gilead Sciences
	Tivicay	Dolutegravir (DTG)	GlaxoSmithKline
Nucleoside reverse transcriptase inhibitors	Emtriva	Emtricitabine (FTC)	Gilead Sciences
	Epivir	Lamivudine (3TC)	GlaxoSmithKline
	Retrovir	Zidovudine (AZT)	GlaxoSmithKline
	Videx EC	Didanosine (ddI)	Bristol–Myers

			Squibb
	Viread	Tenofovir (TDF)	Gilead Sciences
	Zerit	Stavudine (d4T)	Bristol–Myers Squibb
	Ziagen	Abacavir (ABC)	GlaxoSmithKline
Non-nucleoside reverse transcriptase inhibitors	Edurant	Rilpivirine (RPV)	Tibotec Therapeutics
	Intelence	Etravirine (ETR)	Tibotec Therapeutics
	Rescriptor	Delavirdine (DLV)	Pfizer
	Sustiva	Efavirenz (EFV)	Bristol–Myers Squibb
	Viramune XR	Nevirapine (NVP)	Boehringer Ingelheim
Protease inhibitors	Agenerase	Amprenavir (APV)	GlaxoSmithKline
	Aptivus	Tipranavir (TPV)	Boehringer Ingelheim
	Crixian	Indinavir (IDV)	Merck & Co
	Invirase	Saquinavir (SQV)	Hoffmann-La Roche

	Kaletra	Lopinavir + Ritonavir (LPV/r)	Abbott Laboratories
	Lexiva, Telzir	Fosamprenavir (FOS-APV)	GlaxoSmithKline
	Prezista	Darunavir (DRV)	Tibotec Therapeutics
	Reyataz	Atazanavir (ATV)	Bristol–Myers Squibb
	Viracept	Nelfinavir (NFV)	Agouron Pharmaceuticals

Table 1.2: List of all antiretrovirals currently approved by the FDA (www.fda.org) (adapted from (49)).

1.3.1 Nucleoside reverse transcriptase inhibitors (NRTIs)

The first antiretroviral approved for the treatment of HIV infection was Zidovudine (AZT), introduced in 1987. As a nucleoside reverse transcriptase inhibitor (NRTI), AZT (and other drugs of this class) are prodrugs that once phosphorylated by host cell enzymes will bind in the substrate binding site of the RT enzyme and are thus incorporated into the growing DNA strand in the place of a nucleotide. The result is chain termination and inhibition of replication at this step in the viral life cycle (Figure 1.11) (reviewed in (90)). Resistance to NRTIs occurs through two pathways: thymidine analog mutations (TAMs) enhance the enzyme's ability to remove AZT and d4T from the growing DNA chain, while other mutations that confer resistance to the other drugs of this class improve the discrimination between NRTIs and nucleotides of the RT enzyme (91, 92). Other drugs of this class are

summarized in Table 1.2. What became clear after the approval of AZT was that monotherapy for the management of HIV infection was not effective, as many patients began failing treatment with resistance substitutions in the RT enzyme within as little as six months (93, 94). Thus the search for novel therapeutics was spurred.

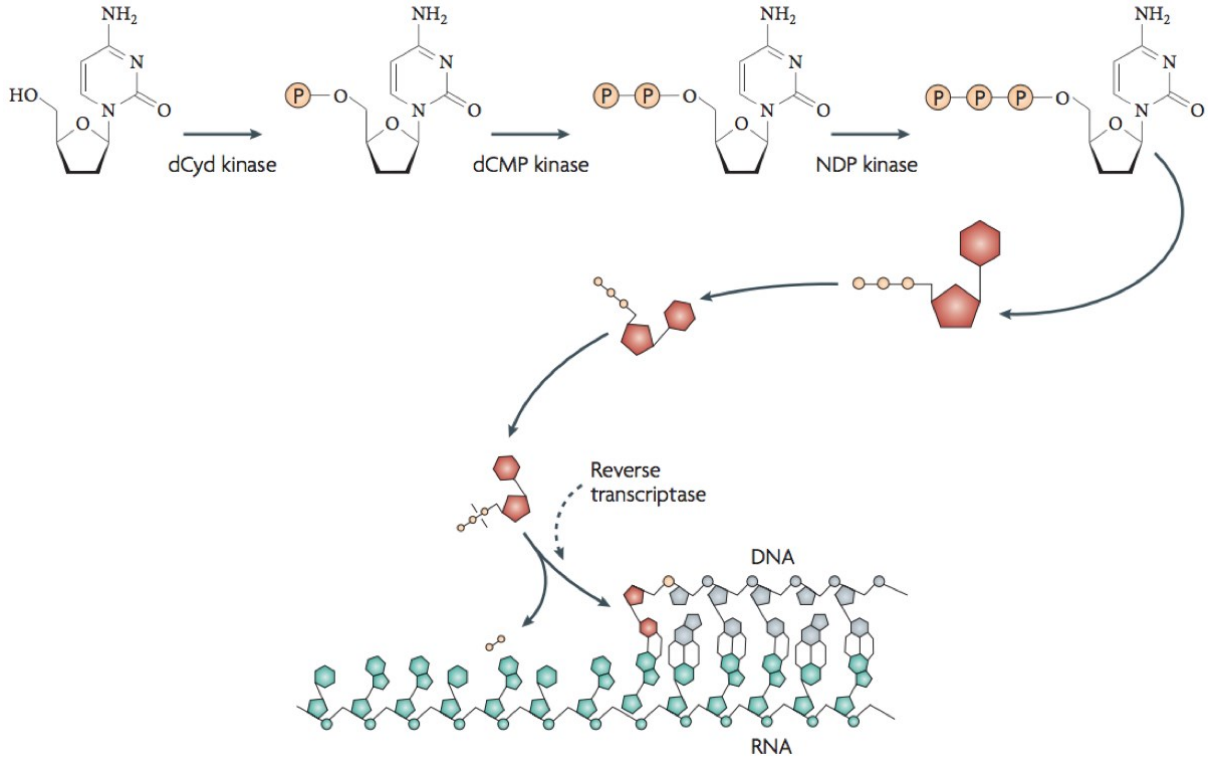


Figure 1.11: Mechanism of NRTI chain termination (adapted from (95)). Conversion of prodrug by cellular kinases is also shown.

1.3.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) hit the market in 1996 with the FDA approval of nevirapine (NVP). Unlike NRTIs, NNRTIs are not competitive inhibitors and bind at a non-catalytic site of HIV RT, causing a conformational change that abrogates the enzyme's activity (reviewed in (96)). However, as is the case with NRTIs, patients treated with NNRTIs are susceptible to virologic failure following the development

of resistance substitutions in the RT enzyme due to the relatively low genetic barrier to resistance of these compounds. Often, single amino acid substitutions are all that is needed for high-levels of resistance, and these substitutions are selected with relative ease *in vivo* (97).

1.3.3 Protease inhibitors (PIs)

The protease inhibitors (PIs) were the third drug class to be approved for the treatment of HIV infection with saquinavir (SQV) in 1995 (98). They act by competitively inhibiting the active site of PR and preventing the maturation of HIV virions by inhibiting Gag and Gag-pol cleavages (see Figure 1.10). These compounds mimic an intermediate formed during the process of proteolysis by binding the catalytic Asp residues at the enzyme's active site (99). PIs generally have a higher genetic barrier to resistance than RT inhibitors, but once resistance to these compounds develops it is usually at a high-level (reviewed in (100)).

1.3.4 Entry inhibitors

Maraviroc (MVC) and enfuvirtide (T-20) are the only two HIV entry inhibitors currently approved for use. MVC does not target a viral factor but a cellular one; specifically, it binds to and inhibits the interaction of CCR5 with the HIV Env protein Gp120 by causing a conformational change in the co-receptor (101). As R5-tropic viruses predominate early in infection, MVC is of use when HIV is diagnosed early, but as the virus tends towards X4-tropism later during the course of infection and progression towards AIDS, this inhibitor becomes less appropriate (102). T-20 is a fusion inhibitor that blocks the fusion of viral and cellular membranes. It is a small, synthetic peptide that prevents the conformational change in the viral Gp41 protein required to bring the two membranes into

close enough proximity for fusion to occur (103). The short half-life of T-20 has limited its usage (104).

1.3.5 Integrase strand transfer inhibitors (INSTIs)

The integrase strand transfer inhibitor (INSTI) class of antiretroviral drugs is the latest to be approved for treatment of HIV-positive individuals. As their name suggest, INSTIs inhibit the second step catalyzed by IN, i.e. strand transfer, through competitive binding to the enzyme's active site. INSTIs not only displace the 3' end of the vDNA from the active site, but also chelate the divalent cation (Mg^{2+} or Mn^{2+}) that is required for IN enzymatic activity (105). There are currently three INSTIs approved for the treatment of HIV infection: raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG) (106). Cabotegravir (CTG) and bictegravir (BIC) are newer INSTIs currently in clinical trials (107, 108).

Although highly efficacious in the management of HIV, both RAL and EVG are susceptible to virological failure through the development of resistance mutations. What is more, most of the changes that cause resistance to RAL also cause resistance to EVG, and vice versa (109). This is, however, not the case with DTG. Not only does DTG appear to have a higher genetic barrier to resistance than either of the other two drugs, it has not yet been shown to definitively select for any resistance-associated changes in treatment-naïve patients (110). Although two recent reports of potential emergence of resistance in individuals treated with DTG in first line therapy recently appeared, baseline IN was not sequenced in one of these cases, nor did the supposed-emergent mutation lead to virological failure while DTG was still being used together with an optimized background regimen (111). The second case reported transient emergence of a T97A substitution

that did not confer any resistance on its own against DTG *in vitro* and was not observed at subsequent time points (112). Although it cannot be excluded that unambiguously documented cases of emergent resistance mutations against first-line DTG will eventually be reported, it is expected that this will be rare. There have also been rare cases of treatment failure with resistance mutations in treatment-experienced but INSTI-naïve patients, and, in this setting, DTG has most often selected for the novel resistance substitution R263K (113). Other substitutions at residues E92, Q148 and N155, have been reported when DTG monotherapy was used in treatment-experienced patients.

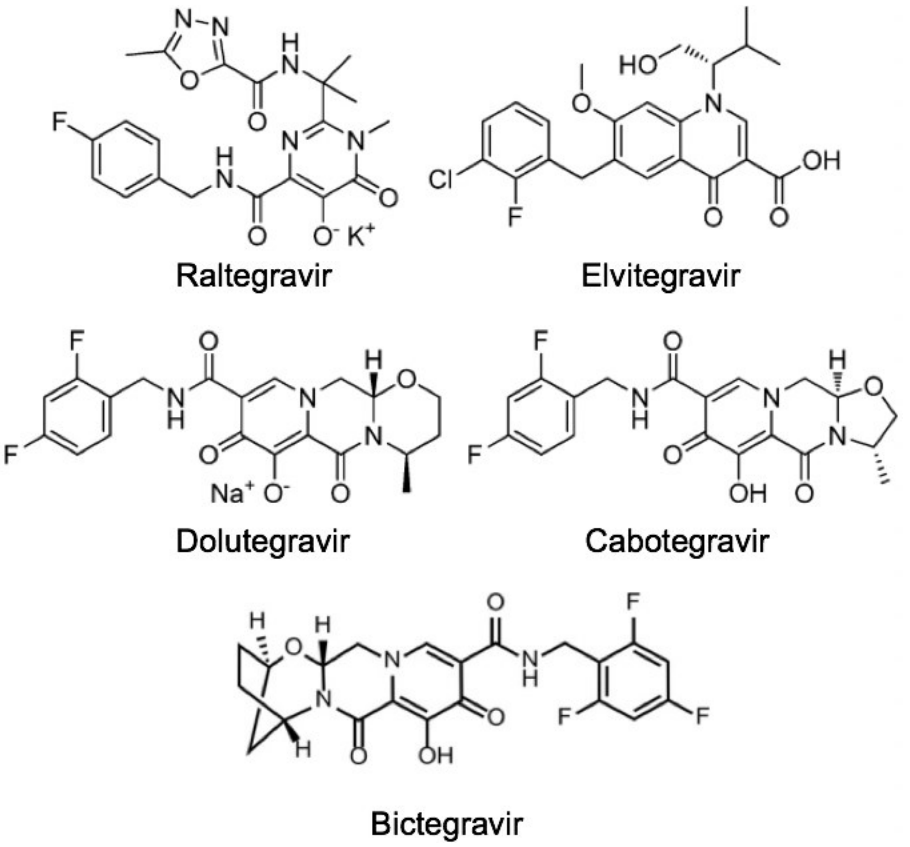


Figure 1.12: Chemical structures of approved integrase strand transfer inhibitors (raltegravir, elvitegravir, dolutegravir) and those currently in clinical trials (cabotegravir and bictegravir) (adapted from (107, 114)).

Primary resistance substitutions arise first in response to INSTI drug pressure and cause a decrease in susceptibility at the expense of viral fitness, most often through alterations to the enzyme's active site where the inhibitors bind (109, 115). Secondary resistance substitutions arise after continued drug pressure and usually act to alleviate the negative effects of primary mutations, and may also increase levels of INSTI resistance (116, 117). Some of these secondary changes are specific to a certain primary resistance pathway, but many may be selected after several different primary mutations.

Pre-clinical and *in vitro* studies have been instrumental in the evaluation of novel therapeutic agents for the treatment of HIV infection, however they do not always accurately predict clinical outcomes for patients. Laboratory viral strains and cell lines, although excellent scientific tools, can never recapitulate *in vivo* human infections with 100% accuracy. In the following sections, we compare both the *in vitro* selection and antiviral activity reported for drugs of the INSTI class with the analogous data available from treated patients to assess the predictive power of *in vitro* studies for INSTI clinical outcomes.

1.3.5.1 Early integrase inhibitors

Viral enzymes have always been attractive therapeutic targets because they often do not resemble cellular proteins and thus non-specific effects are mitigated. Research into IN inhibitors began over 20 years ago with a wide range of candidate compounds, including peptides, DNA binding inhibitors, antimalarial drugs, napthoquinones, flavones, nucleotides, DNA complexes, and multitudes of small molecule inhibitors (49, 118-123). However, none of these candidates ever made it into clinical trials.

The first INSTIs to be described were diketo acid derivatives (DKAs), which were identified by Merck & Co. through a screen of 250 000 compounds. DKAs showed specificity to IN and inhibited viral replication in cell culture in the nanomolar range, but even at this early juncture were subject to viral escape mutations (124). Crystallization of IN with a bound DKA further suggested that these molecules were acting as competitive inhibitors and binding in the enzyme's active site (125).They showed much higher specificity for the inhibition of strand transfer versus 3' processing and, like modern INSTIs, appeared to chelate the two divalent cations that are required for IN enzymatic activity (126). It would later be shown through crystallization of the prototype foamy virus (PFV) IN-DNA complex that this specificity for strand transfer was due to a steric clash between the inhibitor and the 3' dinucleotide, such that INSTIs bind with much lower affinity before 3' processing has occurred (117).

1.3.5.2 Raltegravir

In 2004 a group of researchers at Merck & Co. reported on the efficacy of the diketo acid (DKA)-based lead compound L-870812 against simian immunodeficiency virus (SIV) in infected rhesus macaques (127). This led to the approval of the first INSTI, raltegravir, in 2007 for treatment-experienced AIDS patients with multidrug resistance, and two years later for treatment-naïve individuals as well (49, 128). In the 10 years since its first approval, RAL has been shown to be well tolerated in the vast majority of patients, although it does require twice daily dosing. It displays a modest genetic barrier to resistance, with the most common mutational pathways consisting of changes at positions Y143, Q148, and N155 (129).

The substitutions in IN that have been selected both in cell culture and in treated patients with RAL are summarized in Table 1.3, and the measured fold-changes in resistance to INSTIs for the different combinations of substitutions in each of the major pathways are displayed in Table 1.4. In accordance with previously published studies, the main resistance pathways that have been reported as selected both *in vitro* and *in vivo* with RAL are Y143, Q148, and N155. There were also sporadic reports of changes at positions T66 and E92, mostly *in vitro*. As shown in Table 1.4, these pathways only provide low to moderate changes in RAL susceptibility, which helps to explain their limited selection. Broadly, there is a high concurrence between the resistance pathways selected both in tissue culture under RAL pressure and in patients undergoing therapy with RAL (Table 1.3).

Substitutions	**RAL**		**EVG**		**DTG**		**CTG**		**BIC**	
Selection:	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*
T66										
T66A	X	X	X	X						
T66I			X	X						
T66I/V72A/A128T			X							
T66I/E92Q			X							
T66I/E92Q/T124A			X							
T66I/Q95K/E138K/Q146P/S147G			X							
T66I/T97A/G163R	X									
T66I/T124A			X							
T66I/T124A/Q146L			X							
T66I/I203M		X								
T66I/F121Y/S153Y/D232N			X							
T66I/D232N			X							
T66I/R263K			X							
T66K	X		X	X						
T66K/L74M	X									
T124A/T66K	X		X							
E92										
E92G			X							
E92Q	X	X	X	X	X					

L74M/E92Q		X								
H51Y/E92Q/S147G			X							
H51Y/E92Q/S147G/E157Q			X							
E92Q/M154I	X									
E92V			X							
E92V/T124A			X							
Y143										
Y143C	X	X								
L74M/T97A/Y143R/G163R		X								
T97A/Y143C/G163R		X								
Q95K/Y143C		X								
L74M/T97A/E138D/Y143R/G163N		X								
T97A/Y143C		X								
Y143C/S230R		X								
L74M/T97A/Y143G		X								
Y143H	X	X								
Y143K		X								
T97A/Y143S		X								
T97A/E138A/Y143K		X								
Y143S/V201I		X								
Y143R	X	X								
V72I/Y143R/T206S		X								
L74M/T97A/Y143R		X								
L74M/T97A/E138A/Y143R		X								
T97A/Y143R		X								
Y143R/D232N		X								
G140S/Y143R		X								
Q148										
Q148H	X	X	X	X						
G140S/Q148H	X	X	X	X						
G140C/Q148H	X	X	X	X						
G140A/Q148H	X	X	X	X						
E138K/Q148H	X	X	X	X						
E138A/Q148H	X	X	X	X						
T112S/G140S/Q148H/G163R		X								
E138A/G140S/Q148H		X								
E138A/G140S/Y143H/Q148H		X								
E138K/G140S/Q148H		X								
G140S/Y143H/Q148H		X								
G140S/Q148H/G163R		X								
G140S/Q148H/S230N		X								
Q148K	X	X	X	X						
N17S/Q148K	X									
N17S/Q148K/G163R	X									
G140S/Q148K	X	X	X	X						
G140C/Q148K	X	X	X	X						

G140A/Q148K	X	X	X	X						
E138K/Q148K	X	X	X	X						
E138A/Q148K	X	X	X	X						
Q148K/G163R	X									
E138A/G140A/Q148K		X								
G140C/Q148K/G163R	X									
E138K/Q148K/G163R	X									
E92Q/E138K/Q148K/M154I	X									
G140S/Q148N				X						
Q148R	X	X	X	X				X		
T124A/Q148R	X		X							
H114Y/A128T/Q148R			X							
G140S/Q148R	X	X	X	X						
G140C/Q148R	X	X	X	X						
G140A/Q148R	X	X	X	X						
E138K/Q148R	X	X	X	X						
E138A/Q148R	X	X	X	X						
G140A/Q148R/G163R		X								
Q148R/N155H		X								
E138K/Q148R/G163R		X								
G140S/Q148R/G163R		X								
E138K/Q148R/N155H/G163R		X								
Q148R/N155H/G163R/S230N		X								
L74M/G140A/Q148R		X								
L74M/Q95T/G140A/Q148R		X								
Q148R/D232N	X									
N155										
N155H	X	X	X	X		X				
I60L/T97A/N155H						X				
V72I/N155H		X								
L74M/N155H		X								
L74M/Y143R/N155H		X								
L74M/T97A/V151I/N155H		X								
L74I/V151L/N155H		X								
E92A/N155H		X								
E92Q/N155H		X	X							
E92Q/V151I/N155H		X								
E92Q/N155H/G163R		X								
E92Q/N155H/R263K			X							
Q95K/N155H		X								
T97A/N155H		X								
T97A/Y143C/N155H	X									
T97A/V151L/N155H		X								
T97A/V151I/N155H		X								
T97A/V125A/V151I/N155H		X								
T97A/E138D/V151I/N155H		X								

T124A/V151I/N155H	X									
E138D/N155H		X								
E138K/N155H/G163R		X								
Y143C/N155H		X								
V151I/N155H	X									
V151I/N155H/V125A		X								
V151L/N155H		X								
N155H/G163R		X								
N155H/I204T	X									
N155H/R263K			X							
N155S	X									
N155S/D232N	X									
R263K										
R263K			X		X	X			X	
M50I/R263K					X				X	
A49G/S230R/R263K						X				
M50I/S119R/R263K					X					
H51Y/R263K					X					
S119R/R263K					X					
E138K/R263K					X					
E138AKT/S147G/R263K						X				
V151I/R263K					X					
S153Y/R263K					X					
V260I/R263K						X				
OTHER										
H51Y/G118R					X					
V54I	X									
G59E	X									
L74M		X		X		X				
Q95K	X		X							
T97A		X		X		X				
L101Y/T124A/S153Y					X					
I203M		X								
H114Y			X							
G118R		X			X					
F121Y	X	X	X							
F121Y/G163R	X									
F121Y/D232N	X									
T124A	X		X		X		X			
T124A/P145S			X							
T124A/S153F					X					
T124A/S153Y							X			
T124A/Q146L			X							
T125K		X								
A128T	X		X							

P145S			X							
Q146L							X			
Q146P			X							
S147G			X	X						
V151I	X	X	X			X				
S153Y							X			
M154I		X								
E157Q		X								
I162M							X			
G163E						X				
G163R		X								
Q177R			X							
G193E					X					
S230R	X	X	X							

Table 1.3: *In vitro* and *in vivo* selection of *de novo* INSTI resistance substitutions in HIV IN in tissue culture and in INSTI-naïve individuals. 'X' marks a report of the selection of a substitution or combination of substitutions. Numbers refer to amino acid position in HIV integrase, one letter amino acid code used.
References: (107, 111-113, 130-155)

Genotype	RAL	EVG	DTG	CTG	BIC
T66					
T66A	-	+	-	-	NA
T66A/S153F	-	++	NA	NA	NA
T66I	-	+	-	-	NA
T66I/L74M	+	++	-	-	NA
T66I/E92Q	++	+++	-	-	NA
T66I/F121Y	+	++	NA	NA	NA
T66I/S153Y	-	+++	NA	NA	NA
T66I/E157Q	-	++	-	NA	-
T66I/Q146P	NA	+++	NA	NA	NA
T66I/Q146P/S147G	NA	+++	NA	NA	NA
T66I/Q95K/Q146P/S147G	NA	+++	NA	NA	NA
T66I/Q95K/E138K/Q146P/S147G	NA	+++	NA	NA	NA
T66I/T97A/E157Q	-	++	-	NA	-
T66I/R263K	-	++	-	NA	NA
T66I/E138K/R263K	+	+++	-	NA	NA
T66K	+	+++	-	-	NA
T66K/L74M	++	+++	+	+	NA
E92					
E92G	-	+	-	NA	NA
E92G/S153F	-	+	NA	NA	NA
E92I	-	+	-	-	
E92Q	+	++	-	-	-

V72I/E92Q/E157Q	+	++	NA	NA	NA
E92Q/S147G	-	+	NA	NA	NA
H51Y/E92Q/S147G	NA	+++	NA	NA	NA
H51Y/E92Q/S147G/E157Q	NA	+++	NA	NA	NA
E92Q/E157Q	++	++	-	NA	-
E92Q/R263K	+	+++	-	NA	
E92V	-	+	-	-	NA
Y143					
Y143C	+	-	-	-	-
L68V/Y143C	++	-	-	NA	-
L68V/L74M/Y143C	+++	++	-	NA	-
L74M/T97A/E138A/Y143C	++	NA	-	NA	
Q95K/Y143C	+	-	NA	NA	NA
T97A/Y143C	+++	+	-	NA	-
T97A/Y143C/G163R	++	+	NA	NA	NA
L74M/T97A/Y143G	++	NA	-	NA	NA
Y143H	-	-	-	-	NA
Y143K	+	NA	NA	NA	NA
T97A/E138A/Y143K	++	NA	NA	NA	NA
T97A/Y143S	+	NA	NA	NA	NA
Y143S/V201I	+	NA	NA	NA	NA
Y143R	++	+	-	-	-
V72I/Y143R/T206S	++	NA	NA	NA	NA
T97A/Y143R	+++	++	-	NA	-
L74M/T97A/Y143R	NA	NA	-	NA	NA
L74M/T97A/E138A/Y143R	++		-	NA	NA
L74M/T97A/E138D/Y143R/G163N	+++	++		NA	NA
G140S/Y143R	++	NA	NA	NA	NA
Q148					
Q148H	+	+	-	-	NA
G140S/Q148H	+++	+++	+	+	+
E138K/Q148H	++	++	-	-	NA
L74M/T97A/G140S/Q148H	NA	NA	++	NA	NA
L74M/E138A/G140S/Q148H	NA	NA	++	NA	NA
T97A/E138K/G140S/Q148H/N155H	NA	NA	+++	NA	NA
T97A/T112S/G140S/Q148H	+++	NA	+	NA	NA
T97A/T112S/G140S/Q148H/N155H	+++	NA	+++	NA	NA
E92Q/T97A/G140S/Q148H	NA	NA	++	NA	NA
E138K/G140S/Q148H/N155H	NA	NA	++	NA	NA
T97A/G140S/Q148H	+++	+++	++	NA	+
E138A/G140S/Q148H	+++	+++	++	NA	++
E138A/G140S/Y143H/Q148H	+++		++	NA	NA
E138K/G140S/Q148H	+++	+++	++	NA	+
E138K/G140S/Q148H/M154I	+++	+++	+	NA	NA
V75I/E138K/G140S/Q148H/M154I	+++	+++	+	NA	NA
G140S/Y143H/Q148H	NA	NA	+	NA	NA

G140S/Q148H/N155H	+++	NA	NA	NA	NA
T112S/ G140S/Q148H/G163K	+++	NA	NA	NA	NA
G140S/Q148H/G163K	+++	+++	+	NA	-
Q148K	+++	+++	-	+	NA
G140S/Q148K	+	+++	-	+	NA
E138K/Q148K	+++	+++	++	+++	+
E138K/G140A/Q148K	+++	+++	+++	NA	+++
Q148N	-	+	-	NA	NA
G140S/Q148N	-	+	-	NA	NA
Q148R	++	+++	-	NA	-
T66I/Q148R	++	+++	+	NA	NA
E92Q/Q148R	+++	+++	+	NA	NA
G140S/Q148R	+++	+++	+	+	+
G140S/Q148R/V201I	+++	+++	+	NA	NA
G140C/Q148R	+++	+++	+	++	-
G140A/Q148R	+++	+++	-	NA	-
G140A/Q148R/G163R	++	NA	NA	NA	NA
E138K/Q148R	+++	+++	+	++	-
E138K/G140S/Q148R	+++	+++	+	NA	NA
E138A/Q148R	++	++	+	++	-
N155H/Q148R	+++	+++	+	NA	+
L74I/G140S/Q148R	+++	+++	NA	NA	NA
L74M/G140A/Q148R	+++	+++	++	NA	+
L74M/G140C/Q148R	+++	+++	++	NA	++
E138A/S147G/Q148R	++	+++	-	+	NA
E138K/G140C/Q148R	+++	+++	++	NA	+
N155					
N155H	++	++	-	-	-
T66I/N155H	++	+++	NA	NA	NA
V72I/N155H	++	NA	NA	NA	NA
L74M/N155H	++	++	-	+	-
L74M/V151I/N155H	++	++	NA	NA	NA
L74M/T97A/Y143R/N155H	NA	NA	+	NA	NA
L74M/N155H/R263K	+	+++	+	NA	NA
L74M/T97A/E138A/Y143R/N155H	NA	NA	+	NA	NA
E92Q/N155H	+++	+++	+	+	+
E92Q/V151I/N155H	++	++	NA	NA	NA
E92Q/N155H/G163R	+++	+++	++	NA	+
E92Q/N155H/R263K	++	+++	+	NA	NA
Q95K/N155H	+	++	NA	NA	NA
T97A/N155H	++	++	-	+	-
T97A/V151I/N155H	+++	NA	NA	NA	NA
T97A/V125A/V151I/N155H	+++	NA	NA	NA	NA
T97A/N155H/R263K	+	+++	+	NA	NA
S119R/S147G/V151I/N155H	++	++	-	NA	NA

S119R/T97A/E138K/S147G/V151I/N155H	+++	+++	++	NA	NA
V125A/V151I/N155H	++	NA	NA	NA	NA
E138D/N155H	+	++	NA	NA	NA
Y143H/N155H	++	++	-	+	NA
V151I/N155H	NA	NA	NA	NA	NA
E157Q/N155H	NA	NA	NA	NA	NA
N155H/E157Q/R263K	+	+++	+	NA	NA
N155H/G163K	++	++	-	-	NA
N155H/G163R	++	++	-	-	-
N155H/G163R/R263K	+	+++	+	NA	NA
N155H/D232N	++	++	-	+	NA
N155H/R263K	+	+	+	NA	NA
N155S	+	++	-	-	NA
N155T	+	++	-	-	NA
R263K					
R263K	-	+	+	-	-
M50I/R263K	-	+	+	NA	+
M50I/S119R/R263K	+	++	+	NA	+
H51Y/R263K	-	NA	+	NA	NA
S119R/R263K	-	+	+	NA	+
E138K/R263K	+	+	+	NA	NA
S153Y/R263K	+	++	+	NA	+
OTHER					
M50I	-	-	-	NA	NA
H51Y	-	NA	-	NA	NA
H51Y/S147G	-	+	NA	NA	NA
H51Y/R262K	-	NA	-	NA	NA
V72I	NA	+	NA	NA	NA
V72I/F121Y/T125K	++	++	-	+	NA
V72I/F121Y/T125K/I151V	+	++	-	-	NA
L74M/G118R	++	+	NA	NA	NA
Q95K	+	+	NA	NA	NA
T97A	-	-	-	NA	NA
T97A/F121Y	+++	+++	-	NA	-
L101I	-	NA	-	NA	NA
L101I/S153F	-	-	-	NA	NA
L101I/T124A/S153F	-	-	-	NA	NA
H114Y	-	+	NA	NA	NA
G118R	+	-	+	NA	NA
G118R/E138K	+	-	+	NA	NA
G118S	-	+	-	-	NA
S119R	+	+	-	NA	+
F121Y	++	++	+	-	-
F121Y/T124A	+	+	NA	NA	NA

F121Y/T125K	+	++	-	-	NA
F121Y/G163R	NA	++	NA	NA	NA
T124A	-	-	-	-	NA
T124A/S153Y	-	+	NA	+	NA
T125K	NA	-	NA	NA	NA
A128T	-		-	NA	NA
E138K	-	-	-	NA	NA
G140S	-	+	-	NA	NA
P145S	-	+++	-	-	NA
Q146L	-	++	NA	+	NA
Q146P		+	NA	NA	NA
Q146R	-	+	-	-	NA
S147G	-	+	-	NA	NA
V151I		+	NA	NA	NA
V151L	+	++	+	NA	NA
S153F	-	+	-	NA	NA
S153Y	-	+	+	NA	NA
M154I	-	-	-	NA	NA
E157Q	-	+	-	NA	NA
G193E	-	-	-	NA	NA
S230R	-	NA	-	NA	NA

Table 1.4: Levels of *in vitro* resistance for HIV-1 INSTI mutated viruses. Scale: '-' no fold-change (FC), '+' low FC, '++' moderate FC, and '+++' high FC from measured WT 50% inhibitory concentration (EC_{50}). NA denotes no value available. Numbers refer to amino acid position in HIV integrase, one letter amino acid code used.
References: (107, 109, 130, 131, 133, 134, 138, 142, 145-147, 151-154, 156-171)

The Y143 pathway is specific to RAL; it is not selected by any other INSTI (Table 1.3). This specificity was explained when the crystal structure of prototype foamy virus IN in complex with RAL was solved to show that residue 143 interacts directly with the oxadiazole ring of RAL, forming a π-π stacking interaction that is abrogated when this position is mutated (172, 173). This is in contrast to changes at positions 148 and 155, which disturb the geometry of the IN active site, thereby disrupting the binding of INSTIs (117). Interestingly, levels of resistance conferred by changes at position 143 are variable depending on the specific amino acid change involved (Table 1.4). This phenomenon has been extensively studied and been shown to be true also for the fitness of these variants

(148, 155). The most common substitutions at this position are Y143C and Y143R. They cause low to moderate reductions in RAL susceptibility on their own, but the addition of secondary mutations leads to the levels of resistance being greatly increased (Table 1.4; see also (155)). Although the Y143R pathway provides the highest levels of RAL resistance, it also has a higher genetic barrier to selection, as the amino acid change requires two nucleotide mutations whereas Y143C/H/S only require one change. Both Y143C and Y143H may transition to Y143R and so these substitutions may reflect an intermediary rather than a final selection (155).

There were numerous reports of emergence of substitutions involving position Q148 in response to RAL pressure both in tissue culture and in patient-derived samples, and this is in line with previous studies (140, 174, 175). Early in treatment, substitutions at position Q148 may be seen in isolation, but as they impart a severe fitness cost, they are rapidly compensated for by various secondary resistance mutations as shown in Table 1.3. In growth competition assays, single Q148X mutants showed a significant reduction in fitness compared with WT viruses in the absence of RAL, whereas viruses containing these same mutations outcompeted WT in the presence of the INSTI. Likewise, as secondary mutations are added to Q148X-containing viruses, they outcompete the single mutants whether or not RAL is present in the medium (176). As can be seen in Table 1.4 that as secondary resistance mutations accumulate, the fold-changes in susceptibility to RAL greatly increase as well. This helps to explain why the Q148 pathway dominates in RAL selections.

A N155 pathway is also selected with moderate frequency both *in vitro* and *in vivo* in response to RAL (Table 1.3; see also (109, 177)). This single substitution appears to

have a less deleterious effect in terms of the replication of the virus, and as such is mostly only co-reported with one or infrequently two additional secondary substitutions (176, 177). This observation is also supported by the data collected in Table 1.4: only one or two additional substitutions are required to provide high levels of RAL resistance. In a study examining the evolution of INSTI resistance substitutions in treated patients over time, it was found that mutations at position 155 were often selected earlier during therapy, and then gradually replaced by changes at position 143 or 148 (178). This may be due to higher levels of resistance conferred by the 143/148 pathways as compared to N155H.

1.3.5.3 Elvitegravir

EVG is a monoketo acid derivative that also demonstrated high specificity for inhibition of HIV IN strand transfer reactions (179). EVG was developed by Gilead Sciences and approved for use in HIV infected individuals in 2012 (49). Because EVG is processed by the cytochrome p450 enzyme CYP3A4/5, it needs to be co-formulated with cobicistat to boost plasma concentrations. This permits once daily dosing of EVG (180).

It is evident from both Tables 1.3 and 1.4 that RAL and EVG share both the Q148 and N155 major resistance pathways, although from our literature review it appears that the latter is most often reported for RAL. The data compiled in Table 1.4 clearly show that the levels of resistance conferred by the various mutations of the N155 pathway for EVG are at or above those for RAL and the selection of a greater number of secondary resistance mutations in addition to N155H in patients treated with RAL may be a reflection of this difference. This pathway has also been extensively characterized in terms of EVG resistance by several groups (130, 164, 177). Although some mutants containing the

Y143 pathway displayed moderate levels of EVG, this is most likely due to the secondary resistance mutations present.

The T66 and E92 pathways are predominately selected by EVG, although they do display increased likelihood of selection *in vitro* as opposed to *in vivo* (Table 1.3). As was reported with RAL, there is a dynamism to the temporal selection of EVG resistance mutations that may help to explain these differences. The T66 and E92 pathways are selected earlier under EVG pressure, and are gradually replaced by other pathways, such as Q148X (138, 151, 171). As can be seen in Table 1.4, substitutions in the T66 and E92 pathways provide moderate levels of resistance to EVG, while the Q148 and N155 pathways provide larger fold-changes in resistance. Many more secondary mutations were also reported for T66X and E92X *in vivo* than *in vitro.* While this could be a reporting bias, it could also be reflective of the different requirements for replication under EVG pressure in tissue culture versus in a human host. The S147G pathway is also sometimes selected by EVG in tissue culture and in the clinic but only confers moderate to high levels of INSTI resistance when combined with two or more other resistance substitutions (Tables 1.3 and 1.4).

One of the major limitations for EVG has been that it shares a clinically significant resistance pathway at position 148 with RAL (130). Just as is the case for RAL, significant selection both *in vitro* and *in vivo* of the Q148 pathway in response to EVG was observed (Table 1.3), and this pathway also conferred significant reductions in EVG susceptibility (Table 1.4). Thus, substitutions at position 148, and the accompanying secondary changes, predominate selections with RAL and EVG.

1.3.5.4 Second-generation INSTIs

The relatively low genetic barrier and high degree of cross-resistance among the so called "first-generation" INSTIs RAL and EVG spurred research into the chase for "second-generation" drugs of this class, aimed at retaining efficacy against RAL/EVG resistant variants. There have been four candidate second-generation INSTIs to date. DTG, manufactured by ViiV-Healthcare and GlaxoSmithKline, was approved in 2013 for both treatment-naïve and experienced patients and is the only second-generation INSTI to be approved to date (181). MK-2048 showed potent activity against most RAL/EVG resistant variants and did not select for the same substitutions in tissue culture studies but its clinical development was halted due to poor pharmacokinetics. Both CTG and BIC are promising and both are currently in advanced clinical trials (108, 112, 152, 182).

The resistance profile of DTG has been extensively characterized during the past few years (reviewed in (49, 109, 183)). DTG has been shown to have a longer binding half-life to HIV IN than either RAL or EVG, which may help to explain why it maintains activity against most first-generation INSTI resistant variants (163).

It can be seen in Table 1.3 that DTG only sporadically selects for common first-generation INSTI resistance substitutions and that resistance to this compound most often derives from the DTG-specific R263K pathway. Although R263K was seen rarely as a secondary EVG resistance substitution prior to the approval of DTG, it has since been selected by the latter in tissue culture selection studies, and in four INSTI-naïve but treatment-experienced patients undergoing DTG therapy (107, 110, 135, 137, 171, 177, 184). What is notable about this substitution is that, unlike those discussed for RAL and EVG, the R263K substitution only results in low levels of resistance to DTG. It also has a significant impact on the fitness of the virus, and has yet to be compensated by secondary

resistance mutations in tissue culture selections (135, 185). It has been reported that patients with non-B subtype viruses selected for N155 pathway mutations in response to DTG (Table 1.3; see also (137)). *In vitro*, subtype B viruses harbouring this mutation are sensitive to DTG, so these selections may reflect a subtype-specific difference (159).

There are fewer reports on the resistance patterns of CTG, a novel INSTI under development at GlaxoSmithKline. CTG was derived from DTG and has a longer half-life than its predecessor, which makes it possible to administer it as a long acting injectable for both pre-exposure prophylaxis and treatment of HIV infection (186). In the LATTE clinical trial, one patient in the CTG arm did develop a mutation in the Q148 pathway which suggests that this second-generation INSTI may select for the same mutations as RAL and EVG (108). In *in vitro* selection studies, CTG has selected for changes at positions 146 and 153, that can be rarely selected by EVG and DTG, respectively (Table 1.3).

BIC is a more recent second-generation INSTI and as such there is less information available in regard to resistance against this drug. Tissue culture selection studies with BIC performed by Gilead Sciences selected for the R263K substitution in IN, and at an earlier week than occurred with DTG in parallel studies (107). So far, the results of a phase II trial of BIC in HIV infected individuals has only reported results to 24 weeks, As yet, there has been no detection of resistance-associated changes in IN (112).

As is shown in Table 1.4 and been reported previously, the Q148 pathway seems to confer the highest fold-changes in resistance to second-generation INSTIs upon the addition of at least two secondary mutations, and has been selected in a patient failing CTG-based therapy (107, 108, 131, 162). However, the fold-changes in susceptibility to

second-generation INSTIs are almost always below those that have been observed with RAL and EVG. Table 1.3 notes that the Q148 pathway has yet to be selected for *in vitro* or *in vivo* by DTG or BIC, suggesting that decreases in susceptibility with Q148 may only be worrisome in INSTI-experienced patients with Q148 mutations; neither compound appears to select for this pathway on their own. Of the other first-generation INSTI resistance pathways, only changes associated with position 155 appear to have any effect on the susceptibility of HIV to DTG, CTG, or BIC, and these changes are relatively low-level (Table 1.4).

1.3.5.5 Experienced patients and selections using resistant viruses

Due to the high degree of cross-resistance between RAL and EVG, neither may be used as a salvage therapy for patients failing the other. DTG, however, has been used in select cases in patients failing RAL- or EVG-based therapies. The results of these studies are summarized in Table 1.5. This strategy was first explored in the VIKING phase II clinical trial, in which 27 highly treatment-experienced patients with drug resistant viruses were switched from their RAL-containing regimens to DTG. At week 24 69% of participants achieved undetectable viral loads, as compared to 88% in treatment-naïve patients in the SPRING-2 trial (110, 145). Patients with Q148X + two additional secondary mutations fared the worst in this study, which is in line with the *in vitro* susceptibility assays reported in Table 1.4. Interestingly, patients who experienced failure with DTG in this study did so through the accumulation of several RAL/EVG resistance mutations in addition to those present at baseline, and not through the selection of DTG-specific mutations such as R263K (see Table 1.5). It has been reported that the presence of various first-generation INSTI resistance mutations may be incompatible with R263K (159).

Prev. INSTI	Baseline genotype	Genotype at DTG failure	Ref.
RAL	G140S/**Y143H**/Q148H	**L74I,M/E138A**/G140S/Q148H	(145)
RAL	G140S/Q148H	**L74M,I/T97A**/G140S/Q148H	(145)
RAL	L74M/T97A/ E138A/Y143R	L74M/T97A/E138A/Y143R**/N155H**	(145)
RAL	L74M/T97A/Y143R	L74M/T97A/Y143R/**N155H**	(145)
RAL	G140S/Q148H	**T97A/E138K**/G140S/Q148H/**N155H**	(145)
RAL	E138A/G140S/Q148H	**E92Q/T97A**/G140S/Q148H	(145)
RAL	G140S/Q148H	**E138K**/G140S/Q148H/**N155H**	(145)
RAL	E157Q	E157Q	(187)
RAL	G140S/Q148H/N155H	**T97A/T112S**/G140S/Q148H/N155H/**S230N**	(147)
RAL	S119R/S147G/ V151I/N155H	**A49P/L68F/T97A**/S119R/**E138K**/ S147G/V151I/N155H/**L234V**	(166)
RAL	ND[a]	**G118R**	(136)
EVG	ND[a]	**G118R**[b]	(136)

Table 1.5: IN substitutions in viruses isolated from RAL- or EVG-experienced patients subsequently failing therapy with DTG. Substitutions that differ between baseline and treatment failure are in bold. Numbers refer to amino acid position in HIV integrase, one letter amino acid code used. Raltegravir (RAL), elvitegravir (EVG), dolutegravir (DTG), no change detectable (ND).
[a] Patient had undetectable viral load when switched to DTG monotherapy.
[b] Along with multiple polymorphisms in IN (D10E, E11D, S24D, D25E, N27H, V31I, L45Q, I60IM, V72IL, T112L, T124N, T125A, V126L, R127K, V201I, K215N, I220V, N232D, L234I and D286N).

There have been other, infrequent reports of RAL-experienced patients failing DTG salvage therapy. One such patient failed RAL and subsequently DTG with the only known resistance-associated change being E157Q (187). Although the authors found that the IN derived from this patient was highly resistant to both INSTIs, others showed that the laboratory virus NL4.3 containing E157Q was hyper-sensitive to DTG, highlighting the variability that background changes and polymorphisms may introduce into analyses (161). A different patient failed DTG with a combination of the Q148 and N155 pathways, which is reminiscent of the combinations found in some VIKING patients (147, 188). A

RAL-experienced patient was also reported to have failed DTG with N155 pathway mutations (166).

A G118R mutation was selected by MK-2048 drug pressure, as well as by DTG in certain non-B subtypes of HIV-1 (135, 182, 189). G118R was also shown to be present in two patients, one previously treated with EVG and the other with RAL, during failure on DTG monotherapy (136). The selection of G118R in certain settings and not others is most likely due to codon usage at position 118; although rare in certain subtypes of HIV-1, the presence of the GGA (G) codon is favourable to a transition to AGA (R).

Analogous to these sporadic reports of INSTI-experienced patients subsequently failing DTG-based therapies, tissue culture selection studies have used HIV with INSTI resistance mutations to mimic the situation seen in some patient populations. The results of these studies are summarized in Table 1.6. When viruses with mutations of the Q148 pathway are placed under DTG pressure, they select for additional secondary resistance mutations, similar to what was reported in the patients in the VIKING clinical trial. As expected, viruses containing other primary INSTI resistance substitutions can acquire secondary INSTI resistance mutations under continued selection with either RAL or EVG (Table 1.6; see also (154)). After 30 weeks of DTG selection, viruses that contained E92Q or N155H at baseline selected for R263K (159). In another study that lasted only 6 weeks, additional substitutions to E92Q, Y143R or N155H were not detected (154).

Starting genotype	Drug selection	Genotype (week)	Reference
E92Q	DTG	E92Q (8), E92Q/**R263K** (30)	(154, 159)
E92Q	RAL	E92Q, **L74M**/E92Q (8)	(154)
E92Q	EVG	E92Q (8)	(154)
E138K	RAL	**T66I/T97A**/E138K/**P142T/G163R** (30)	(190)
E138K	EVG	**G70R**/E138K/**N155H/V249I/R263K** (30)	(190)

Y143C	RAL	**Y143R, Y143R/G163R, E92Q/Y143R, G163R/E170A** (8)	(154)
Y143R	DTG	Y143R (8)	(154)
Y143R	RAL	Y143R, **L74M**/Y143R, Y143R/**N155H** (8)	(154)
Q148K	DTG	**E138K**/Q148K (8)	(154)
Q148K	RAL	Q148K, **E138K**/Q148K (8)	(154)
Q148K	EVG	Q148K, **E138K**/Q148K (8)	(154)
Q148R	DTG	ND (30)	(159)
Q148R	DTG	**G140S**/Q148R, **G140S**/Q148R/**V201I**, **E138K/G140S**/Q148R (8)	(154)
Q148R	RAL	Q148R, **G140S**/Q148R, **G140S**/Q148R/**V259I**, **L74M/G140S**/Q148R (8)	(154)
Q148R	EVG	Q148R, **E138K**/Q148R (8)	(154)
Q148H	DTG	**G140S**/Q148H, **T97A/G140S**/Q148H, **V75I/E138K/G140S**/Q148H/**M154I** (8)	(154)
Q148H	RAL	**G140S**/Q148H	(154)
Q148H	EVG	**G140S**/Q148H	(154)
G140S	DTG	**V131I/V54I/Q148R**/G140S (30)	(159)
N155H	DTG	N155H (8), N155H/**R263K** (30)	(154, 159)
N155H	RAL	N155H, **G70R**/N155H, N155H/**G163R/D232N**, **S119R**/N155H, **P142T**/N155H/**G163R** (8)	(154)
N155H	EVG	N155H, N155H/**S230K**, N155H/**D232N**, N155H/**E170K**, **G70R/V75I**/N155H (8)	(154)
R263K	RAL	R263K (30)	(190)
R263K	EVG	**M50I/T66I**/R263K (30)	(190)
G118R	DTG	**T66I**/G118R/**E138K** (25)	
G118R	RAL	**T66I**/G118R/**E138K** (30)	(190)
G118R	EVG	**T66I**/G118R/**E157Q** (30)	(190)
H51Y	DTG	H51Y/**R262K** (25)	(191)
H51Y	RAL	H51Y/**G140S/Q148R** (30)	(190)
H51Y	EVG	H51Y/**T66I/S147G/G163R/E170K/D232N** (30)	(190)
G140S/Q148R	DTG	**K14R/H51Y/V54I**/G140S/Q148R (30)	(159)
E92Q/N155H	DTG	E92Q/N155H (30)	(159)
E138K/R263K	RAL	**H51N/T66I/T97A/S119R**/E138K/**Y143H**/R263K (30)	(190)
E138K/R263K	EVG	**M50I/T66I/S119R**/E138K/**S147G**/R263K (30)	(190)
H51Y/G118R	DTG	H51Y/G118R (25)	(191)
H51Y/G118R	RAL	H51Y/**T66I**/G118R (30)	(190)
H51Y/**G118R**	EVG	H51Y/**T66I/S147G/H171R/D232N** (30)	(190)
H51Y/R263K	DTG	H51Y/**E138K**/R263K (25)	(191)
H51Y/R263K	RAL	**E138K/Y143R**/R263K (30)	(190)
H51Y/R263K	EVG	**V31I**/H51Y/**E92Q**/R263K (30)	(190)

Table 1.6: *In vitro* selections using INSTI resistant viruses. Substitutions that differ between baseline and final selection are in bold. Numbers refer to amino acid position in HIV integrase, one letter amino acid code used. Raltegravir (RAL), elvitegravir (EVG), dolutegravir (DTG), no change detectable (ND).

Attempts to select further changes to DTG-specific resistance pathways have yielded more nuanced results. R263K-containing viruses are sensitive to RAL and unable to select for additional changes under pressure by this compound unless secondary mutations such as H51Y or E138K are also present. R263K, however, readily selects for EVG resistance (Table 1.6). This is in line with previous data that identified R263K as a secondary EVG resistance mutation (171). G118R readily selects both primary and secondary resistance mutations under pressure with all three INSTIs, with or without the initial presence of additional secondary mutations. Lastly, the DTG resistance mutation H51Y facilitates the emergence of other resistance-associated substitutions in selections with all three INSTIs, in agreement with its previously characterized role as a secondary change (169).

1.3.5.6 Discussion

For the first-generation INSTIs RAL and EVG, the resistance pathways that were selected *in vitro* were generally predictive of the mutations that would arise in patients failing therapy with these drugs, although the frequencies of primary and/or secondary mutations selected may vary depending on whether *in vitro* or *in vivo* results are considered. The picture is not so straight-forward for the newer INSTIs. Data from patients are largely non-available in regard to newer INSTIs such as CTG and BIC. Since CTG has selected for the Q148 pathway *in vivo,* it is possible that the clinical resistance profile of this INSTI will resemble that of the first-generation INSTIs. However, since CTG did not select RAL or EVG resistance pathways in tissue culture, the situation may be more complex. BIC has

so far selected for the same substitutions *in vitro* as DTG and this suggests that this compound might also select for similar pathways as DTG in patients.

Although the most common substitution selected *in vitro* by DTG (R263K) has also been the most common pathway seen in patients failing DTG, other aspects of tissue culture selection studies with DTG have not been as predictive. One third of all INSTI-naïve patients reported to have failed DTG to date (2/6) have done so with the N155 pathway, even though the N155H substitution alone does not cause large fold-changes in DTG resistance in *in vitro* assays (Table 1.4). Part of the explanation may be that the majority of selection studies and *in vitro* INSTI resistance testing has been performed with subtype B HIV-1. Indeed, the two patients who developed N155H in response to DTG both had non-B viruses. In culture selection studies, non-B viruses predominantly selected the G118R substitution and N155H was not observed (135). This shows a divergence between the *in vivo* and *in vitro* resistance profile of DTG.

In the case of the two INSTI-experienced patients who failed DTG monotherapy with the G118R mutation, the effect of polymorphisms and subtype differences on the selection of INSTI resistance may be important, as the GGA glycine codon can more easily transition to AGA, explaining why arginine is present at a higher frequency in non-B subtypes (136). Many of the secondary resistance mutations listed in Tables 1.3 and 1.4 occur at positions that are considered polymorphic, i.e. dependent on subtype and geographical distribution; this complicates the nature of the selection of these polymorphic changes as a function of INSTI exposure. Any description of transmitted INSTI drug resistance (for a review of the effect of subtype diversity and polymorphisms on HIV-1 INSTI resistance see (139)) must also be thereby complicated. A more accurate

portrait of patterns of second-generation INSTI resistance mutations in non-B subtypes, which is increasingly important as access to these medications increases in developing countries, will require that selection studies be conducted more frequently with non-B primary isolates (192).

The Q148 pathway remains the dominant route to INSTI resistance, regardless of the individual compound used. All second-generation INSTIs show lower activity against HIV as secondary mutations of this pathway accumulate, and the results of INSTI-experienced patients on DTG therapy suggest that once present, the sequential selection of further mutations in this pathway will result in greatly diminished susceptibilities to this compound (Table 1.5; see also (188)). Even though the Q148 substitutions may not be selected by either DTG or BIC *in vivo*, they remain important for the future of these compounds.

Predictions of which pathways will be important for resistance to second-generation INSTIs, unlike first-generation INSTIs, may not easily follow from *in vitro* studies. If resistance to these compounds turns out to be due to random genetic changes, that are not easily predicted, genotyping of patient-derived viruses may not be able to determine treatment success when these compounds are employed (139, 188). Perhaps, the high genetic barrier to resistance of DTG will forces HIV to evolve along different mutational pathways *in vitro* versus *in vivo*, depending on the subtype of the virus and the baseline polymorphisms that are present.

1.4 Cure research

Although HAART has transformed a diagnosis of HIV infection from a death sentence into a chronic, manageable disease, integration of the viral genome into the host DNA has meant that infection persists for life. Patients must remain adherent to their treatments or risk reactivation of HIV viruses from the latent reservoir, as well as the development of drug resistance (reviewed in (193, 194)). Hypothetically, there could be two "levels" of HIV cure: a functional cure wherein the latent reservoir may not be completely eliminated but HIV replication is undetectable without the need for therapy, and a sterilizing cure that requires the complete elimination of HIV from the body (194).

Because HIV infects a multitude of cells of the immune system, cure strategies are complicated by the long-lasting resting phenotype of many of these cells. For antiretroviral therapy to be effective, a cell should be activated and HIV replication must be occurring, as all ARVs inhibit a specific step in the viral life cycle. A popular strategy that is currently being investigated by many different groups is termed "Shock and Kill." It involves using so-called latency reactivating agents (LRAs) that stimulate the transcription of the HIV LTR from resting cells ("shock") that can then be targeted by ARVs and eliminated ("kill") (193). One such LRA that is under investigation is vorinostat (SAHA), an HDACI inhibitor that has been approved by the FDA for the treatment of certain cancers (195). Histone deacetylation is associated with closed chromatin and low levels of transcription (70). Inhibition of deacetylation has been shown *in vitro* and in HIV-infected individuals to increase transcription of HIV RNA (196). Unfortunately, in the clinical trials that have so far been reported with SAHA, no diminution in the viral reservoir was seen (197). Other LRAs include other HDAC inhibitors such as benzamides, short-chain fatty acids, cyclic tetrapeptides, as well as protein kinase C, IL-7, IL-15, TLR3, TLR7, and TLR9 agonists,

disulfiram, interferon-α, anti-programmed-death receptor 1(PD-1)/anti-PD-1 ligand monoclonal antibodies, and janus kinase (JAK) and mammalian target of rapamycin (mTOR) inhibitors (reviewed in (198)).

As mentioned above, HIV usually favours the CCR5 co-receptor early in infection (26). Interestingly, there is a circulating polymorphism in the CCR5 gene among people of Western European descent that includes a deletion of 32 nucleotides from CCR5 (termed CCR5Δ32) that renders these individuals quite refractory to HIV infection (199). In fact, the one documented case of HIV "cure" occurred when Timothy Ray Brown (also known as the Berlin Patient) received a bone marrow transplant from a CCR5Δ32 donor (200). However, doctors have tried to repeat this result with the so-called Boston Patients, and although they did achieve undetectable levels of HIV RNA, both subjects did eventually experience HIV viral rebound (201). This was most likely due to the fact that these two individuals received transplants from a CCR5 wild-type donor, underscoring the importance of CCR5Δ32, and not only an allogenic stem cell transplant as in the success of the Berlin patient. CCR5Δ32 is currently being investigated under multiple strategies to become part of the HIV cure armament, but because of the high costs and risks associated with bone marrow transplants and gene therapy, it is not likely to achieve widespread use, even if successful (202).

1.5 Vaccines

With no cure and the issues inherent with taking medication daily for a lifetime (namely toxicity and tolerability), a vaccine against HIV infection is sorely needed. Two types of vaccines are possible: preventative (blocks infection) and therapeutic (treats infection),

and both have remained elusive to HIV researchers for decades. HIV has evolved numerous ways to evade the host's immune system, and can infect the cells of said system, which makes vaccine development extremely complicated (203). Another issue complicating HIV vaccine development is the lack of a correlate for protective immunity (reviewed in (204)).

Since the beginning of the HIV/AIDS pandemic in the 1980s, there has been immense interest in the procurement of a vaccine, and as such many candidates have undergone clinical trials. The vast majority of trials showed no difference between treatment and placebo groups, and some even saw an increase in infection rates in the vaccinated population, likely due to increased activation of T cells (reviewed in (205)). The only trial to show positive efficacy for preventing HIV infection to date was the RV144 trial, with a vaccine efficacy at 31.2%. Many studies are currently underway to examine correlates of protective immunity in patients from this trial, as well as ways to expand the efficacy of this vaccine. Many clinical trials with therapeutic HIV vaccine candidates have also been conducted but have been associated with limited clinical benefit (reviewed in (206)). Broadly neutralizing antibodies towards the Env glycoproteins are also currently a fashionable area of HIV vaccine and therapeutic research (reviewed in (207)).

1.6 Thesis rationale

The INSTI DTG appears to have a higher genetic barrier to resistance than its predecessors RAL and EVG; it also retains activity against most RAL and EVG resistant HIV variants (reviewed in (109, 208)). In the rare instances of DTG failure, most often the novel resistance substitution R263K is selected for in patients, and this is also the

predominant mutation seen in *in vitro* selections with this compound (113, 135). This substitution results in low-level resistance to both DTG and EVG, at the expense of ~30% viral replication capacity and IN activity (135).

Common RAL/EVG primary resistance mutations occur within the CCD of IN and lead to resistance by disrupting the orientation of the enzyme active site and/or interfering with the binding of these competitive inhibitors (183). Residue 263, however, is located in the CTD of IN and causes resistance through a hitherto unexplored mechanism. The CTD of IN is involved in interactions with host cell proteins, DNA binding, IN multimerization, and nuclear import of the HIV PIC (reviewed in (46)). If the R263K substitution is not directly affecting the interaction of DTG with the active site of IN, it is possible that it provides low-levels of resistance through an indirect effect on one or more of these processes.

Specifically, residues K264 and K266 are acetylated by p300 and deacetylated by HDACI (recruited to IN by KAP1) (67, 73). This post-translational modification of IN increases DNA binding, strand transfer activity, and viral infectivity generally. These residues have also been implicated in protein multimerization and nuclear import, but it is unclear whether acetylation is a factor in these processes (61, 71). By introducing an amino acid change adjacent to these two important residues, the R263K substitution may affect binding of p300 and/or KAP1 to IN, alter their abilities to post-translationally modify the CTD, or may even be acetylated itself. Any or all of these changes could have an impact on HIV-1 IN biology.

In the VIKING clinical trial, RAL-experienced patients failing therapy with primary INSTI resistance substitutions were placed on DTG salvage therapy. Although these

individuals saw lower success rates than other patient populations on DTG, there was no selection of the signature R263K substitution. Instead, an accumulation of secondary first-generation INSTI resistance mutations was seen in patients that subsequently failed DTG in this trial (145). If the R263K substitution truly represents a novel mechanism of INSTI resistance, then there is the possibility that it would be compatible with the pathways that mediate RAL and EVG resistance; however, R263K was not selected in the VIKING trial. This raises the possibility that primary RAL/EVG resistance mutations preclude the emergence of R263K.

1.7 Thesis objectives

The purpose of this thesis was threefold. First, the mechanism through which the R263K substitution provides resistance to DTG was to be investigated. Second, establishing an alternative resistance mechanism for R263K, assessment of whether this pathway was compatible with those which provide resistance to RAL and EVG was to be performed. Third, showing that DTG and first-generation INSTI resistance pathways were not mutually exclusive, and the potential impact of the combination of these pathways to produce a highly DTG resistant HIV variant was to be explored.

1.7.1 Specific objective 1

The first specific objective of this thesis was to determine the mechanism of resistance of the R263K substitution. Because of the proximity of R263K to two residues that are post-translationally modified during the course of HIV infection, the effect of this substitution on the process of IN acetylation was examined using co-immunoprecipitation studies, biochemical analyses of IN acetylation, as well as short- and long-term infectivity studies

utilizing inhibitors of acetylation and deacetylation. This portion of the thesis is presented in Chapter 2 and has been submitted for publication.

1.7.2 Specific objective 2

The second objective of this thesis was to evaluate whether or not the R263K substitution is compatible with the most common primary resistance substitutions that arise during treatment with RAL and EVG. The impacts of the combination of these mutations on IN enzymatic activity, viral infectivity, and resistance to INSTIs were evaluated and are summarized in Chapter 3. This portion of the thesis has previously been published (159).

1.7.3 Specific objective 3

Having shown in Chapter 3 that the N155H and R263K resistance pathways were compatible, the third specific objective of this thesis was to determine whether the combination of known INSTI resistance pathways with R263K could lead to the production of a highly DTG resistant variant. N155H and R263K were combined with the most common secondary resistance mutations associated with N155H during RAL/EVG treatment failure, and the effects on enzymatic activity, viral infectivity, and resistance to INSTIs were investigated. These results are presented in Chapter 4 and have been previously published (209). In Chapter 5 this objective was continued through the evaluation of the polymorphic substitution E157Q in combination with R263K. This substitution had been associated with resistance to RAL and EVG in the past, and was now implicated in a clinical failure with DTG and as such was an interesting avenue for the promotion of R263K-mediated DTG resistance (132, 187). As this position had also been identified as a putative interactor with the DNA binding inhibitor FZ41, DNA binding activities and resistance to FZ41 are also reported, along with IN strand transfer activity,

viral infectivity, and resistance to INSTIs of WT, E157Q, R263K, and E157Q/R263K variants. These results have been published previously (161).

Chapter 2

HIV-1 resistance to dolutegravir is affected by cellular histone acetyl-transferase activity

This chapter was adapted from the following manuscript submitted for publication in PLOS Pathogens in 2017: “HIV-1 resistance to dolutegravir is affected by cellular histone acetyl-transferase activity” by Kaitlin Anstett, Thibault Mesplede, and Mark A. Wainberg. K.A. performed approximately 90% of the experiments and data analysis while T.M. and M.A.W. supervised and advised the project. K.A., T.M., and M.A.W. wrote the manuscript.

2.1 Abstract

Integrase strand transfer inhibitors are the newest class of antiretrovirals to have been approved for the treatment of HIV. Canonical resistance to these competitive inhibitors develops through substitutions in the integrase active site that disrupt drug-protein interactions. However resistance against the newest integrase inhibitor dolutegravir (DTG) is associated with a R263K substitution at the C-terminus of integrase that causes resistance through an unknown mechanism. The integrase C-terminal domain is involved in many processes over the course of infection and is post-translationally modified via acetylation of three lysine residues, that are important for enzyme activity, integrase multimerization, and protein-protein interactions. Here, we report that regulation of the acetylation of integrase is integral to the replication of HIV in the presence of DTG and that R263K specifically disrupts this regulation, likely due to enhancement of interactions with the histone deacetylase I complex, as suggested by co-immunoprecipitation. Although no detectable differences were observed in levels of cell-free acetylation of wild-type (WT) and mutated R263K enzymes, the inhibition of cellular histone acetyl-transferase enzymes sensitized NL4.3$_{WT}$ viruses to DTG while NL4.3$_{R263K}$ was almost completely unaffected. When levels of endogenous acetylation were manipulated in virus-

producing cells, inhibitors of acetylation enhanced the replication of $NL4.3_{R263K}$ whereas inhibition of deacetylation greatly diminished the replication of $NL4.3_{WT}$. Taken together, these results point to a pivotal role of acetylation in the resistance mechanism of HIV to some second-generation integrase strand transfer inhibitors such as DTG.

2.1.2 Importance

This is, to our knowledge, the first report of the influence of post-translational modifications on HIV drug resistance. Both viral replication and resistance to second-generation integrase strand transfer inhibitors of both WT and INSTI-resistant HIV were differentially affected by acetylation, likely as a result of altered interactions between integrase and the cellular deacetylation machinery. Many “shock and kill” strategies to eradicate HIV manipulate endogenous levels of acetylation in order to reactivate latent HIV. However, our results suggest that some drug resistant viruses may differentially respond to such stimulation, which may complicate the attainment of this goal. Our future work will further illuminate the mechanisms involved.

2.2 Introduction

Human immunodeficiency virus (HIV) has claimed the lives of over 35 million people worldwide since the beginning of the epidemic. Despite the advent of effective antiretroviral medications (ARVs), drug resistance remains a prime concern in the fight against acquired immunodeficiency syndrome (AIDS) (88, 210). The integrase strand transfer inhibitors (INSTIs) represent the most recently approved drug class for the treatment of HIV infection. They act to inhibit the second reaction catalyzed by the HIV integrase enzyme (IN or INB to denote subtype B), i.e. the insertion of the viral DNA

genome (vDNA) into the cellular chromatin, an essential step in the viral replication cycle (105). Raltegravir (RAL) was approved by the United States Food and Drug Administration in 2007 followed by Elvitegravir (EVG) in 2012 (211, 212). Although highly effective, both drugs are susceptible to the rapid emergence of drug resistance substitutions within IN in treated patients. Moreover, most of these substitutions provide cross-resistance against both compounds (109).

In 2013, Dolutegravir (DTG) was approved for use in treatment-naïve individuals and has a higher genetic barrier to the selection of resistance than RAL/EVG and retained activity against most RAL/EVG resistant viruses (213). Indeed, instead of selecting for resistance substitutions that disrupt the active site of IN to which INSTIs bind, such as RAL and EVG, DTG has not yet selected for resistance-associated substitutions in treatment-naïve patients (214). In treatment-experienced patients and in tissue culture, DTG selected for the novel R263K substitution which led to a low-fold change in the 50% inhibitory concentration (IC_{50}) for DTG and diminished viral replicative capacity (135, 137). Located in the C-terminus of the enzyme, R263K potentially causes resistance through a different mechanism than other INSTI resistance substitutions. However, as the C-terminus of IN is an unstructured region and the crystal structure of HIV IN remains elusive, homology modeling has been unable thus far to rule out an interaction between the R263K substitution and the catalytic core domain of the protein (169) (215).

The IN protein can be post-translationally modified by various host proteins (63). Specifically, IN is known to be acetylated both *in vitro* and *in vivo* by the cellular histone acetyl-transferase (HAT) enzymes p300 and GCN5 at residues K264, K266, and K273; this acetylation enhances DNA binding, enzyme activity, and integration (67, 68). It has

also been shown that a protein termed KAP1 recruits the histone deacetylase (HDAC) I complex to HIV IN, catalyzing its deacetylation and decreasing integration rates (73). Residues K264 and K266 have also been implicated in viral pre-integration complex (PIC) nuclear import and the multimerization of IN (62, 72). The present study was designed to evaluate whether the R263K substitution affected the acetylation of nearby residues and/or whether the regulation of this post-translational modification might be a cause of R263K-mediated DTG resistance.

Here, we have used multiple approaches to show a lack of detectable differences between levels of acetylation of INB_{WT} and INB_{R263K}. However, when cells where infected with $NL4.3_{WT}$ and subsequently treated with HAT inhibitors (HATi), a significant decrease in the IC_{50} for DTG occurred that was not observed for $NL4.3_{R263K}$. Similar patterns were observed for other second-generation INSTIs, i.e. cabotegravir (CTG) and bictegravir (BIC). In contrast, HIV inhibition by RAL was unaffected by the use of HATi. Consistently, $NL4.3_{WT}$ virus produced under conditions of HDAC inhibition showed a reduced peak of replication while $NL4.3_{R263K}$ was unaffected. Conversely, HATi increased the peak of replication for $NL4.3_{R263K}$ but did not affect $NL4.3_{WT}$. Lastly, co-immunoprecipitation assays showed that INB_{R263K} bound with more affinity to KAP1 than INB_{WT} while binding to p300 was unaffected. Thus, WT and R263K-containing HIV-1 appear to react in opposite ways to modulation by acetylation, which may be due, at least in part, to an altered interaction with KAP1 and a subsequent increase in the deacetylation of INB_{R263K}.

2.3 Materials and methods

2.3.1 Experimental design

The research objectives of this study were to evaluate whether the R263K substitution affected the acetylation of the C-terminus of the HIV integrase protein, and whether the resulting aberrant post-translational modifications might be a mechanism of resistance to DTG.

2.3.2 Cells and reagents

PM1, 293T and TZM-bl reporter cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Marvin Reitz, from Dr. Andrew Rice and Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. and cultured as reported previously (135). TZM-bl and 293T cells were maintained in Dulbecco Modified Eagle medium (DMEM) and PM1 cells in RPMI (Gibco); both were supplemented with 10% foetal bovine serum, 50 IU of penicillin/mL, 50 µg of streptomycin/mL, and 2 mM L-glutamine at 37ºC in 5% CO_2. Merck & Co., Inc., Gilead Sciences, Inc., ViiV Healthcare Ltd., GlaxoSmithKline Plc., and Toronto Research Chemicals Inc. supplied raltegravir, bictegravir, dolutegravir, lamivudine, and cabotegravir, respectively. Anacardic acid, C646, and vorinostat were obtained from Sigma Aldrich Co.

2.3.3 [H^3] Acetylation assay

The generation of the pET15b$_{WT}$ and pET15b$_{R263K}$ plasmids using site-directed mutagenesis has been described previously (169). Recombinant integrase proteins were expressed in BL21 (DE3) bacterial cells and purified as published (135). Integrase reactions were assembled on ice in 10% glycerol, 50mM HEPES pH 8.0, 1mM DTT, 10mM sodium butyrate, 0.1 nmol integrase, 17 pmol p300 (Enzo Life Sciences), and between 0 and 20 nmol [H^3]-labelled acetyl-CoA (Perkin Elmer) in a final volume of 20 µL per reaction. Reactions were incubated at 30°C for 45 mins, after which then 50 µL 25%

TCA was added and counts per minute were measured after an additional 30 mins incubation at 4°C using the Millipore multiscreen filtration plate system (Cat No: MSFCN6B 50) as per manufacturer's instructions.

2.3.4 Co-immunoprecipitation

The pACGFP-1C$_{IN(WT)}$ plasmid was obtained from Xiao-Jian Yao at the University of Manitoba and the pACGFP-1C$_{IN(R263K)}$ was created as described in (135). 293T cells were transfected with either expression construct and cells were collected after 24h into 1mL ice-cold PBS and spun at 500x g for 3 mins at 4°C. Cells were washed twice with 500 µL PBS, then lysed in 200 µL RIPA buffer (10mM Tris/Cl pH 7.5, 150mM NaCl, 0.5mM EDTA, 0.5% NP-40, protease inhibitor cocktail pellet (Sigma Aldrich), 1mg/mL Dnase I (Invitrogen), 2.5mM $MgCl_2$), placed on ice and pipetted vigorously every 10 mins for 30 mins. Samples were spun at maximum speed for 20 mins at 4°C, then lysates were diluted in 300 µL wash buffer (10mM Tris/Cl pH 7.5, 150mM NaCl, 0.5mM EDTA). 25 µL Chromotek GFP-trap_A beads (Chromotek, Munich) per sample were vortexed and diluted in 500 µL wash buffer, then spun at 2.5 x1000 g for 2 mins at 4°C. This wash was repeated twice before cell lysates were added to beads and samples were incubated with inversion for 1h at 4°C. Samples were then spun at 2.5 x1000 g for 2 mins at 4°C, supernatants were discarded and beads were washed three times with 500 µL wash buffer. Proteins interacting with the beads were then separated by SDS-PAGE and used for further analysis.

2.3.5 Western blot analysis

Acetylation assays were performed as described above with the exception that non-radiolabelled acetyl-CoA was used and reactions were stopped by freezing at -20°C for

30 mins. 4% SDS-PAGE loading dye (200mM Tris-Cl pH6.8, 400mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol) was added to Co-IP eluates or acetylation reactions, then boiled at 95°C for 10 mins and run on a 12% SDS-page protein gel. Western blots were performed on a PVDF membrane (BioRad). Primary antibodies used were: Acetyl-lysine (mouse; Thermofisher), HIV-1 integrase (rabbit; NIH), KAP1 (rabbit; Abcam), and p300 (mouse; EMD Millipore). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Thermofisher.

2.3.6 MTT cytotoxicity assay

The cytotoxic concentrations of anacardic acid, C646, and vorinostat in TZM-bl cells were determined as described in (216). Briefly, compounds were serially diluted 1:10 and then added to TZM-bl cells in a 96-well plate. Cells were incubated for 48h at 37°C, then 10 µL 5% MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Invitrogen, Cat. No.: M-6494) in PBS was added, cells were incubated at 37°C for 3h prior to the addition of 110 µL lysis buffer (10% (v/v) Triton X-100 in acidified isopropanol) and shook overnight in the dark at room temperature. Absorbance at 570nm (540nm and 690nm) was determined using a microplate reader.

2.3.7 Generation of NL4.3 HIV-1 viral clones

The generation of the pNL4.3$_{IN(WT)}$ and pNL4.3$_{IN(R263K)}$ plasmids by site-directed mutagenesis has been reported previously (135). Similar methods were used to generate the pNL4.3$_{IN(G140S/Q148H)}$ plasmid through site-directed mutagenesis using the following primers: G140S-sense (5'-GGGGATCAAGCAGGAATTTAGCATTCCCTACAATC-3') and G140S-antisense (5- GATTGTAGGGAATGCTAAATTCCTGCTTGATCCCC-3'), and Q148H-sense (5'-CTACAATCCCCAAAGTCACGGAGTAATAGAATCTATG-3') and

Q148H-antisense (5'-CATAGATTCTATTACTCCGTGACTTTGGGGATTGTAG-3'). Genetically homogenous viral stocks were produced as described previously (135). Briefly, 293T cells were transfected with 12.5 µg of the various pNL4.3 plasmids using Lipofectamine 2000. At 4h after transfection, media were changed to Opti-MEM + 10% FBS ± 10mM anacardic acid, 10µM C646 or 0.5 µM vorinostat. At 48h after transfection, cell culture supernatants were collected and filtered at 0.45µm to remove plasmids and cell debris, then HAT/HDAC inhibitors were filtered out (if necessary) using Centrifree ultracentrifugation devices (EMD Millipore Cat No: 4104). Viruses were aliquoted and stored at -80˚C. Viral stocks were quantified by measuring cell-free reverse transcriptase (RT) activity in culture fluids.

2.3.8 HIV susceptibility to integrase strand-transfer inhibitors

HIV susceptibilities to DTG, RAL, lamivudine (3TC), CTG, and BIC were measured by the infection of 30,000 TZM-bl cells using 100,000 RT units per well of each virus in the presence of 1:10 (RAL, DTG, 3TC) or 1:4 (CAB, BIC) serial dilutions of drugs. After 48h, cells were lysed and luciferase production was measured using the Luciferase Assay System (Promega, Madison, WI, Canada). For synergy assays, anacardic acid, C646, or vorinostat were also serially diluted 1:10 2h post-infection and added to cells. To investigate the effect of HAT/HDAC inhibitors on ARV IC_{50} values, 10mM anacardic acid, 10 µM C646, 0.5 µM vonrinostat, or DMEM (control) was added at 2 or 12h post-infection.

2.3.9 Long-term infectivity assay

PM1 cells were diluted to 20 000 cells/well in a 96-well plate, then infected with 150 000 RT units per well of WT and mutant NL4.3 viruses that were produced under various

conditions in triplicate per plate per assay. Samples were collected at days 3, 5, 7, 11, and 14 post-infection for RT quantification.

2.3.10 HIV infectivity and replication capacity

HIV-1 infectivity was measured through the infection of 30,000 TZM-bl cells per well using serial 1:4 dilutions of the various NL4.3 viral clones. Levels of infection were measured as described above.

2.3.11 Protein expression and purification

Plasmids were maintained and purified from *Escherichia coli* strain XL10-Gold ultracompetent cells (Stratagene), while proteins were expressed from BL21(DE3) Gold cells. Luria-Bertani (LB) broth (Multicell), was supplemented with 100 µg/mL ampicillin and used for bacterial culture. Expression and purification of IN proteins were performed as previously described with some modifications (217). Fractions containing purified integrase were dialyzed into storage buffer (20 mM HEPES, 1 M NaCl, 1mM EDTA, 5 mM DTT, 10 % glycerol, pH7.5), aliquoted and stored at -80ºC.

2.3.12 Statistical analysis

Each experiment is an average of at least two replicates performed in triplicate (n=6). 50% effective concentration (EC_{50}), 50% inhibitory concentration (IC_{50}), 50% cytotoxic concentration (CC_{50}), and 95% confidence intervals were calculated using Prism 7.0 Software and all figures were created using this same software. Student's *t*-tests were performed using the OpenEpi toolkit, accessible freely online at www.openepi.com.

2.4 Results

2.4.1 *In vitro* acetylation of HIV-1 integrase

The R263K substitution introduces a lysine residue into a domain of IN that can be acetylated both *in vitro* and *in vivo* (67, 68). We thus investigated whether the R263K substitution can interfere with IN acetylation, since the latter provides an additional lysine substrate for the histone acetyl transferase p300. To test this, we measured levels of acetylation of the WT and R263K-containing IN proteins in cell-free acetylation assays using p300 in the presence of [H^3]-labelled acetyl-CoA. The results are summarized in Fig 1. No differences in the radiolabelling of IN were detected in experiments performed with a gradient of acetyl-CoA (Fig 2.1A) or at a fixed concentration (Fig 2.1B). We next investigated the cell-free acetylation of IN proteins by western blot analysis using a primary anti-acetylation antibody and were again unable to detect differences between INB_{WT} and INB_{R263K} (Fig 2.1C). It is worth noting that various IN oligomers of different sizes are visible in Figure 1C after SDS-PAGE under denaturing conditions.

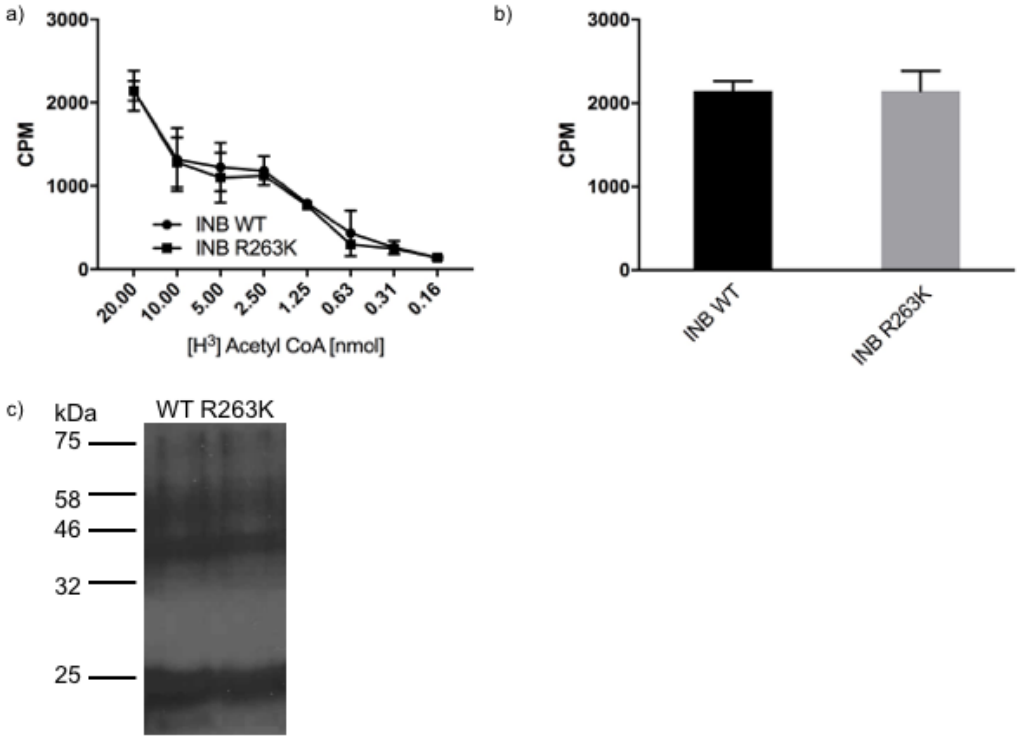


Figure 2.1: The R263K substitution does not diminish IN acetylation in cell-free assays. Acetylation of HIV-1 integrase protein was measured as counts per minute (CPM) of [H3]-labelled acetyl-CoA. A) CPM as a function of [H3] acetyl-CoA concentration. B) CPM at a 20 nmol concentration of [H3] acetyl-CoA. Mean ± SD. C) Acetylation assay by Western blot. Acetylation of HIV-1 integrase protein as measured by Western blot with α-acetyl-lysine primary antibody in a 12% SDS-PAGE gel.

2.4.2 Modulation of acetylation affects INSTI resistance in tissue culture

Given that (1) IN acetylation contributes to DNA binding and that (2) INSTIs are competitive IN inhibitors, we hypothesized that acetylation might influence HIV susceptibility to INSTI inhibition in tissue culture. To study this, we used a variety of small chemical compounds to interfere with cellular acetylation and deacetylation and probed the impact of this on HIV-1 infectivity. We first used the MTT assay as previously described (216) to determine the 50% cytotoxic concentration (CC_{50}) in TZM-bl cells of anacardic acid (AA), an allosteric inhibitor of p300, C646, a competitive p300 inhibitor (collectively termed 'HATi'), and vorinostat (a pan-HDAC inhibitor, referred to as SAHA). The results are summarized in Table 2.1. The effects of these drugs on the ability of HIV-1 to infect TZM-bl cells were also tested (Fig 2.2). Using both of these assays, CC_{50} drug concentrations that had little or no cytotoxic effects on HIV-1 infectivity in TZM-bl cells were determined for use in further experiments.

Inhibitor	CC_{50}	95% Confidence Intervals
Anacardic acid (AA)	47.44 mM	41.89 - 53.73
Vorinostat (SAHA)	3.974 µM	3.015 - 5.238
C646	35.38 µM	28.24 - 44.32

Table 2.1: 50% cytotoxic concentrations of inhibitors used in this study. Summary of 50% cytotoxic concentrations (CC_{50}) values for each inhibitor in TZM-bl cells as measured using the MTT assay with 95% confidence intervals listed.

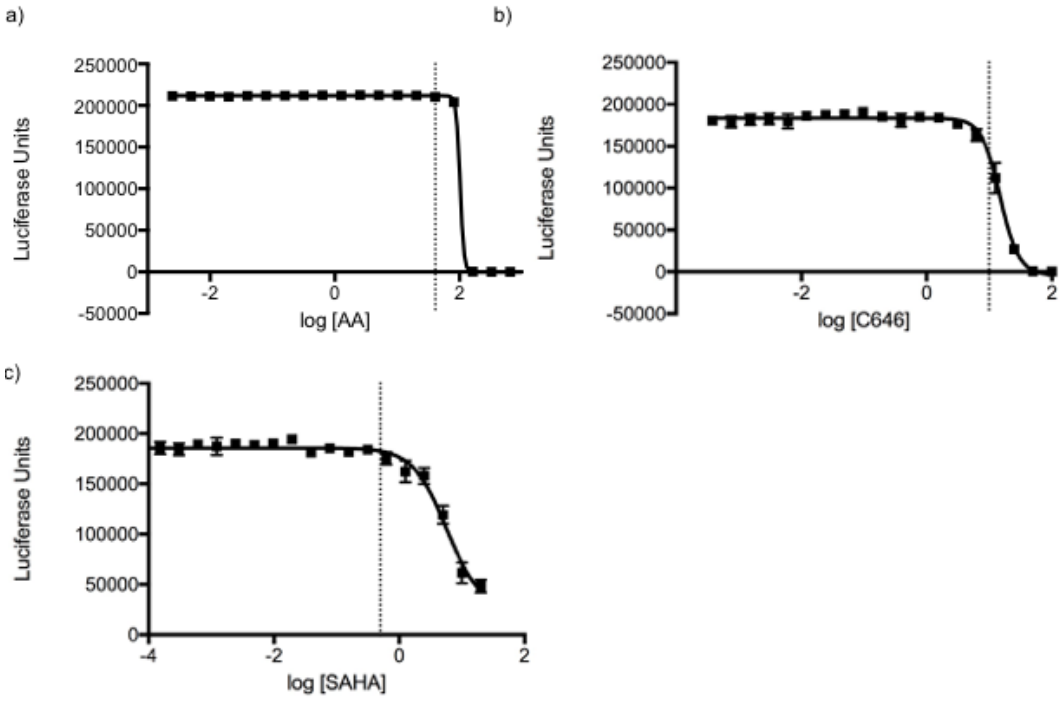


Figure 2.2: The modulation of acetylation in TZM-bl cells does not affect luciferase production at non-cytotoxic concentrations. Titration of a) anacardic acid (AA), b) C646, or c) vorinostat (SAHA) in TZM-bl cells infected with NL4.3WT. Infectivity of virus over drug concentrations measured by luciferase production at 48h post-infection. Dotted line shows highest concentration of each drug used in subsequent assays. Mean plotted (n=6), error bars represent ± SD.

To verify that modulation of INSTI susceptibility by acetylation specifically affected integration, we measured the DTG susceptibility of NL4.3 viruses in the presence of HATi or SAHA either 2 or 12 hours post-infection. As seen in Fig 2.3 and summarized in Table 2.2, the addition of HATi at 2 hours post-infection to the NL4.3$_{WT}$ infection resulted in a lower IC_{50} for DTG compared to the addition of SAHA or the no drug control. It is possible that the modulation of cellular acetylation would also affect later stages in the virus's lifecycle, such as the activity of the transactivating viral protein Tat, and that this could confound our observations of decreased infectivity upon HATi. However, this result was not observed when HATi was added at 12 hours post-infection. Moreover no such

decrease in IC_{50} was seen for $NL4.3_{IN(R263K)}$ at any time point. There was, however, a trend towards sensitization with HATi for the $NL4.3_{IN(G140S/Q148H)}$ mutant (a common combination of RAL resistance substitutions).

Time of addition:	**2 hours post-infection**				**12 hours post-infection**	
Genotype	+AA	+C646	+SAHA	NA	+AA	NA
WT	**0.41 (0.31 - 0.55)***	**0.34 (0.17 - 0.67)***	0.71 (0.50 - 1.01)	1.00 (0.56 - 1.80)	1.14 (0.86 - 1.51)	1.00 (0.80 - 1.24)
R263K	0.94 (0.64 - 1.39)	0.79 (0.54 - 1.15)	1.19 (0.70 - 2.01)	1.00 (0.68 - 1.48)	**1.77 (1.39 - 2.26)***	1.00 (0.74 - 1.35)
G140S/ Q148H	0.67 (0.46 - 0.98)	0.57 (0.36 - 0.92)	0.82 (0.52 - 1.28)	1.00 (0.50 - 1.99)		

Table 2.2: Changes to the relative dolutegravir 50% inhibitory concentrations when acetylation or deacetylation is inhibited 2 or 12 hours post-infection. Relative 50% inhibitory concentrations (rIC_{50}) values for dolutegravir (DTG) in TZM-bl cells treated with either 10 mM anacardic acid (AA), 10 μM C646, 0.5 μM vorinostat (SAHA), or no inhibitor control (NA) 2 or 12 hours post-infection normalized to NA for each virus with 95% confidence intervals listed in brackets. * denotes rIC_{50} values that are significantly different from the control by Student's *t*-test. ($p \leq 0.05$).

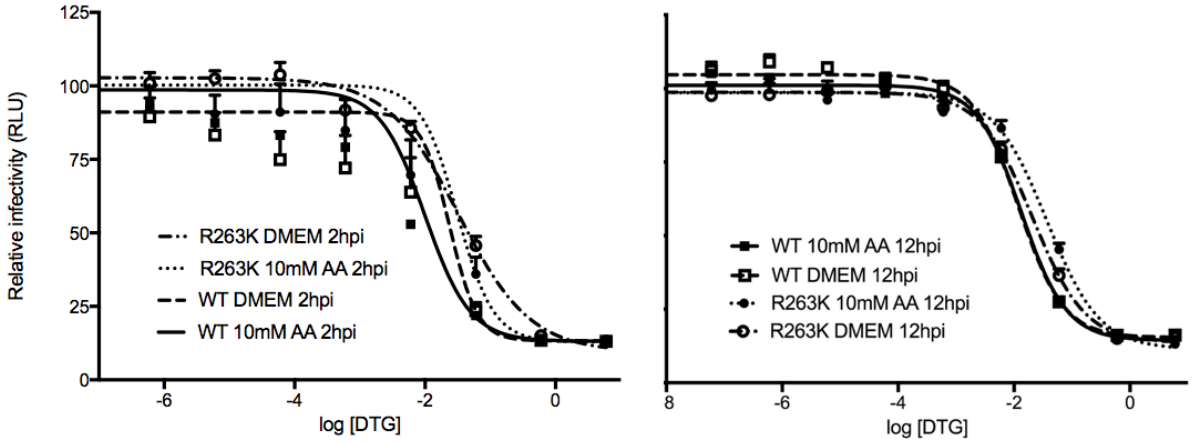


Figure 2.3: Inhibition of acetylation sensitizes WT HIV to DTG when added early during infection. NL4.3 infectivity was measured by luciferase luminescence and normalized to the no drug control for each virus. 10 mM Anacardic acid (AA) was added a) at 2 hours post-infection (hpi) or b) 12 hpi. Mean plotted (n>6), error bars represent ± SD.

There was a general lack of effect on HIV-1 susceptibility to lamivudine (3TC) and RAL (Table 2.3). For CTG and BIC, two novel INSTIs currently in clinical development, the effects were mixed. AA had no effect on HIV-1 susceptibility to CTG or BIC, whereas C646 sensitized NL4.3$_{WT}$ and NL4.3$_{IN(G140S/Q148H)}$ to both drugs and NL4.3$_{IN(R263K)}$ hypersensitized to BIC but not to CTG.

ARV	**3TC**				**BIC**				**CTG**				**RAL**			
Geno-type	+A	+C	+S	-	+A	+C	+S	-	+A	+C	+S	-	+A	+C	+S	-
WT	**1.45 (1.35 - 1.55) ***	0.76 (0.66 - 0.87)	1.17 (0.90 - 1.52)	1.00 (0.79 - 1.25)	0.96 (0.64 - 1.46)	**0.25 (0.14 - 0.43) ***	0.68 (0.29 - 1.75)	1.00 (0.68 - 1.54)	1.12 (0.77 - 1.62)	**0.19 (0.15 - 0.27) ***	1.19 (0.58 - 2.38)	1.00 (0.73 - 1.38)	1.21 (0.936 - 1.57)	0.80 (0.50 - 1.27)	0.90 (0.61 - 1.32)	1.00 (0.65 - 1.54)
R263K	1.02 (0.82 - 1.25)	**0.56 (0.44 - 0.72) ***	1.17 (0.98 - 1.38)	1.00 (0.80 - 1.26)	0.78 (0.57 - 1.07)	**0.23 (0.13 - 0.43) ***	0.90 (0.54 - 1.49)	1.00 (0.63 - 1.60)	1.41 (1.06 - 1.84)	1.59 (1.03 - 2.44)	0.71 (0.46 - 1.68)	1.00 (0.60 - 1.68)	0.71 (50.12 - 1.02)	0.54 (0.30 - 0.97)	1.00 (0.66 - 1.50)	1.00 (0.71 - 1.40)
G140S/Q148H	1.37 (1.08 - 1.72)	0.57 (0.69 - 1.12)	**2.14 (1.55 - 2.94) ***	1.00 (0.74 - 1.36)	1.13 (0.76 - 1.68)	**0.37 (0.21 - 0.67) ***	1.00 (0.72 - 1.38)	1.00 (0.66 - 1.51)	1.06 (0.75 - 1.49)	**0.45 (0.28 - 0.72) ***	0.98 (0.67 - 1.43)	1.00 (0.71 - 1.41)	~1†	~1†	~1†	~1†

Table 2.3: Changes to the relative 50% inhibitory concentrations for 3TC and INSTIs when acetylation or deacetylation is inhibited 2 hours post-infection. Summary of relative 50% inhibitory concentrations (rIC$_{50}$) of select antiviral drugs in TZM-bl cells treated with either anacardic acid (+A), C646 (+C), vorinostat (+S), or no inhibitor control (-) normalized to '-' for each virus with 95% confidence intervals listed in brackets. * denotes rIC$_{50}$ values that are significantly different from the control by Student's *t*-test. ($p \leq 0.05$). † NL4.3$_{IN(G140S/Q148H)}$ RAL IC$_{50}$s were all > 5 μM (NL4.3$_{WT}$ IC$_{50}$ = 55.75 nM).

2.4.3 Disruption of acetylation in producer cells differentially affects HIV replication and drug resistance

Having determined in single cycle replication assays that HATi sensitized WT but not R263K-containing HIV to DTG, when added early but not late during infection, we next investigated whether a disruption of acetylation in viral producer cells might also influence HIV replication. 293T cells were transfected with either NL4.3$_{WT}$ or NL4.3$_{IN(R263K)}$ in the

presence of HATi, SAHA, or a no drug control. When viral stocks were collected, they were filtered to remove all inhibitors, diluted to 100 000 RT units and used to subsequently infect TZM-bl cells. While no effect was seen on the IC_{50} of DTG (data not shown), SAHA significantly decreased the infectivity of WT viruses but had no effect on R263K viruses. In contrast, C646 had the opposite effect, i.e. the infectivity of $NL4.3_{INR263K)}$ was increased two-fold without any significant effect on the infectivity of $NL4.3_{WT}$ (Table 2.4).

Geno-type	**$NL4.3_{WT}$**				**$NL4.3_{IN(R263K)}$**			
Condition	+AA	+C646	+SAHA	NA	+AA	+C646	+SAHA	NA
Relative EC_{50}	**1.66 (1.42 - 1.94)***	0.95 (0.84 - 1.07)	**3.18 (2.74 - 3.70)***	1.00 (0.76 - 1.31)	0.77 (0.70 - 0.86)	**0.40 (0.36 - (0.46)***	1.31 (1.19 - 1.44)	1.00 (0.65 - 1.53)

Table 2.4: Differences in the relative infectivity seen for HIV-1 viruses transfected under the inhibition of acetylation or deacetylation. 50% effective concentration (EC_{50}) values for NL4.3 viruses transfected from 293T cells treated with either anacardic acid (+AA), C646, vorinostat (SAHA), or the no inhibitor control (NA), normalized to the no drug control with 95% confidence intervals listed in brackets. * denotes EC_{50} values that are significantly different from the control for each virus by Student's *t*-test. ($p \leq 0.05$).

Single cycle infectivity assays do not always recapitulate prolonged effects relating to viral infectivity. Therefore, we also infected PM1 cells with the various filtered virus preparations at 150 000 RT units per well and quantified RT enzyme activity as a function of virus production over the course of 14 days. A peak of replication under all conditions for both viruses was seen at day 5 post-infection. However, the viruses responded differently to HATi or SAHA treatment, with SAHA reducing the peak of replication for $NL4.3_{WT}$ but not $NL4.3_{IN(R263K)}$ whereas HAT inhibition had no effect on $NL4.3_{WT}$ but greatly enhanced peak replication for $NL4.3_{IN(R263K)}$ (Figure 2.4). These results are consistent with those of the infectivity assays in TZM-bl cells as summarized in Table 2.4.

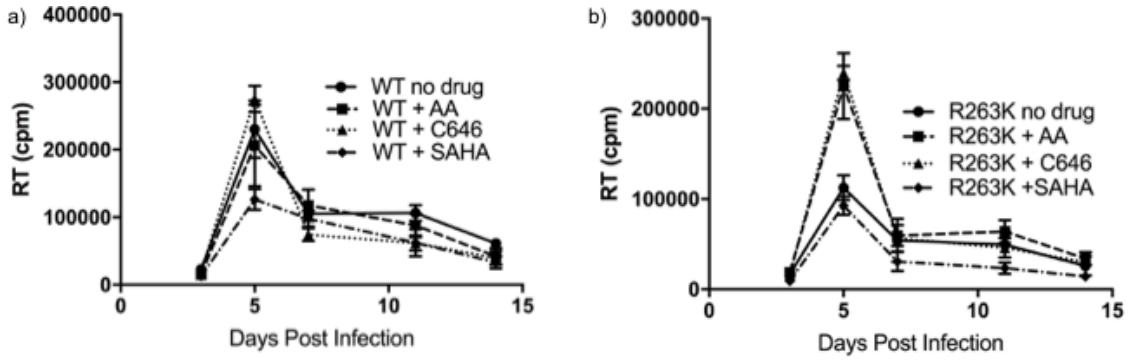


Figure 2.4: WT and R263K-containing NL4.3 viruses produced in the presence of HATi/SAHA respond in opposite ways over the course of long-term infection. a) NL4.3WT and b) NL4.3R263K. Reverse transcriptase (RT) activity in counts per minute (CPM) plotted (n>3), error bars represent ± SD.

2.4.4 The R263K substitution increases IN binding to the HDAC 1 complex protein KAP1

HIV integrase has been shown to interact with and be modified by a number of host cell proteins (reviewed in (63)). Because proper acetylation is important for IN enzymatic activity, and the inhibition of HATs and HDACs have such markedly different effects on WT versus R263K-containing virus, we investigated whether the INB_{R263K} protein interacted with the components of the cellular acetylation machinery differently than INB_{WT}. Figure 2.5 shows the results of a co-immunoprecipitation experiment using 293T cells transfected with GFP-INB constructs. The results show that INB_{R263K} pulled down more KAP1/TRIM28 than did INB_{WT}, while no difference in interactions with p300 were observed. Accordingly, the differential response of WT and R263K-containing HIV-1 to modulation of cellular acetylation may be due, at least in part, to altered interactions with the HDAC I complex.

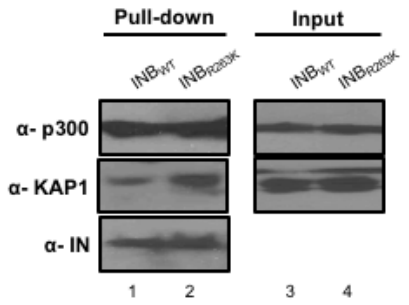


Figure 2.5: HIV-1 integrase with the R263K substitution has a higher affinity for Kap1. Co-immunoprecipitation of GFP-tagged integrase (GFP-INB) from 293T cells. Left panel: GFP-INB was immunoprecipitated from transfected 293T cells using α-GFP agarose beads. Both p300 and KAP1 co-purified. Right panel: Input of cellular lysates.

2.5 Discussion

This is, to the best of our knowledge, the first report of drugs targeting post-translational modifications in a manner that can have a significant effect on HIV drug resistance. We have demonstrated that inhibiting p300 can change the level of resistance against certain INSTIs and that these variations depend on specific integrase substitutions. Modulation of acetylation universally sensitized $NL4.3_{WT}$ to second generation INSTIs but not $NL4.3_{R263K}$. It was also observed that disruption of cellular acetylation in virus-producing cells had marked effects on long-term infectivity that were specific to the viral strain tested. We have also shown that the mutant HIV IN protein containing the R263K substitution associated with DTG resistance interacts more favourably with KAP1 than does INB_{WT}.

Previous work showed that acetylation of lysine residues at the C-terminus of HIV IN enhanced enzyme activity both *in vitro* and *in vivo* (67). It has been shown for many DNA binding proteins and transcription factors that acetylation of lysine residues enhances their interactions with DNA (70). Hence, we hypothesized that the R263K substitution, which lies proximal to the acetylated residues K264 and K266, might be able to interfere with the acetylation of IN because of its additional lysine or through altered

interactions with cellular proteins that bind to this domain. Our results suggest that K263 is not a major substrate for acetylation in cell-free assays (Fig 2.1). However, we have shown an enhanced interaction between HIV INB_{R263K} and KAP1 compared to WT integrase. KAP1 has previously been shown to bind preferentially to acetylated IN and to recruit the other components of the HDAC I complex to deacetylate the protein, however whether the interaction we observed is due generally to increased acetylation of the C-terminus, or the specific amino acid change, is unknown (73). This enhanced interaction with the mutated enzyme suggests that INB_{R263K} may be more readily deacetylated than INB_{WT} which should decrease its DNA binding activity and overall enzyme proficiency, consistent with previous findings (135, 169).

Why decreased DNA binding would be selectively advantageous in the presence of DTG is unclear and is the subject of current research in our laboratory. Perhaps spatial or temporal regulation of integrase acetylation within infected cells plays a role in viral infectivity and that altering this regulation creates a superior ability for the R263K-containing virus to replicate in the presence of DTG. Clearly, when cellular acetylation was inhibited we witnessed an increased sensitivity to second-generation INSTIs for WT virus; therefore, acetylation must play a role in the interaction between HIV and these inhibitors. This phenomenon seems to be specific to second-generation INSTIs, as there was no decrease in susceptibility to RAL under the same conditions. The RAL-resistant virus G140S/Q148H behaved similarly to WT virus in these assays, in contradistinction to R263K viruses in the presence of DTG and CTG. This demonstrates that these effects are specific for IN and were not caused by broad modulation of cellular processes. The

fact that the R263 is located close to acetylated residues provides a potential explanation for this specificity.

The dysregulation of cellular acetylation in virus-producing cells also had marked effects on the long-term infectivity of HIV. HDACi reduced the peak of replication for WT but not R263K-containing HIV, while HATi reciprocally enhanced the peak of replication for $NL4.3_{IN(R263K)}$ but had no effect on the replication of $NL4.3_{WT}$. HDAC inhibitors are well known as latency reversing agents and have been widely used in so called “shock and kill” strategies to reactivate and purge the latent reservoir, as HDAC-mediated chromatin silencing perpetuates HIV LTR downregulation (reviewed in (218)). Here we show that HDAC activity may also be important for production of infectious WT virions, although the mechanism through which this occurs requires further investigation. These results suggest again that lack of acetylation is advantageous for the survival of the R263K virus and that this effect is exerted both in single cycle and protracted infections.

In contrast to results obtained with CTG and DTG, both WT and R263K-containing HIV were sensitized to BIC when p300 was inhibited. This is interesting because BIC has selected for the R263K substitution in *in vitro* studies and our own analysis in Table 2.4 shows it causes modest resistance to this compound (107). Therefore, although we have shown a definite link between INSTI resistance and cellular acetylation, this may not be the sole mechanism through which the R263K substitution confers resistance against BIC. A possible explanation is that small differences in the ability of BIC, CTG and DTG to bind to the integrase catalytic pocket might render them more or less susceptible to IN acetylation. Future in silico docking, co-crystallization, or cryo-electron microscopy may provide further information on this topic (215, 219, 220).

This study did have its shortcomings. First, we were unable to definitely determine whether or not the R263K substitution was itself acetylated or whether acetylation of IN affected the inhibitor dissociation rate from the IN-DNA complex (163). Further investigation is currently underway to pinpoint the exact cause of our observed results through use of mass spectrometry analysis and the study of IN-DNA interactions using multiple C-terminal mutants. We have also previously reported an effect of the R263K substitution on IN DNA binding activity in cell-free assays (135, 161). While this may also be considered a mechanism through which the substitution provides resistance, it is important to note that these studies were performed with recombinant enzymes purified from *Escherichia coli*, and thus most likely lack proper post-translational modifications (221). Thus the affect of acetylation on IN DNA binding may not have been accurately captured in the previous reports.

Taken together, our results suggest a fundamental role for the acetylation of the integrase protein in HIV resistance against INSTIs and raise two additional questions. First, future studies should examine the feasibility of combining HAT/HDAC inhibitors with INSTI-based therapy for the treatment of HIV infection. Second, “shock and kill” studies should carefully take into account the nature of the ART regimen, as considerations of acetylation could conceivably affect the treatment outcomes.

2.6 Acknowledgements

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Chapter 3

Dolutegravir resistance mutation R263K cannot coexist in combination with many classical integrase inhibitor resistance substitutions

This chapter was adapted from the following manuscript published in the Journal of Virology in 2015: “Dolutegravir resistance mutation R263K cannot coexist in combination with many classical integrase inhibitor resistance substitutions” by Kaitlin Anstett, Thibault Mesplede, Maureen Oliveira, Vincent Cutillas and Mark A. Wainberg. K.A. performed approximately 80% of the experiments and data analysis, partially supervised by T.M. and aided by V.C. as a trainee under her supervision. M.O. performed *in vitro* selection experiments and M.A.W. supervised and advised the project and obtained funding for its pursuit. K.A., T.M., and M.A.W. wrote the manuscript.

3.1 Preface

In Chapter 2, we examined the mechanism of R263K-mediated DTG resistance. We next sought to investigate whether such resistance was compatible with canonical RAL and/or EVG resistance pathways. When DTG is used in INSTI-naïve but treatment-experienced patients, classical INSTI resistance mutations, such as those that arise under RAL or EVG pressure, do not emerge. Instead, the R263K mutation is seen in a few rare circumstances whereby patients do not respond optimally to therapy (113). However, when INSTI-experienced patients with primary INSTI resistance mutations are placed on DTG as a salvage therapy, R263K does not develop; these patients instead experience the sequential development of secondary INSTI resistance mutations, and it is this accumulation of alterations in the IN coding sequence that leads to resistance to DTG and all other INSTIs, and treatment failure (145). We wished to examine why the R263K mutation does not develop in these INSTI-experienced patients and whether the presence of primary INSTI resistance mutations precludes the development of R263K. To this end,

used both biochemical IN protein expression and tissue culture viral infectivity assays in which we combined R263K together with the four most common RAL/EVG resistance substitutions and assessed the effect of these combinations on enzyme activity, viral infectivity, and drug resistance. We also used cord blood mononuclear cells to perform selections with DTG using HIV-1 subtype B harbouring the different primary INSTI resistance substitutions in order to investigate the probability of the selection of R263K in these backgrounds. We hypothesized that the combination of two primary resistance mutations in a single protein would lead to a larger defect in enzyme activity, and correspondingly viral replication, while possibly conferring increased INSTI resistance.

3.1.2 Abstract

Although the integrase strand transfer inhibitors (INSTIs) raltegravir (RAL) and elvitegravir (EVG) are susceptible to virological failure due to the emergence of drug resistance mutations, the newer INSTI dolutegravir (DTG) has yet to select a resistance mutation in treatment-naive HIV-positive individuals. We have previously hypothesized that resistance against DTG may not be possible if this drug is used in first line therapy because the mutations associated with resistance against it may severely compromise viral replication capacity. In contrast, two INSTI-naive, treatment-experienced individuals have developed a R263K mutation after virological rebound; this mutation had previously been selected and characterized by our lab on the basis of DTG studies in cell culture. Moreover, patients who previously failed RAL or EVG with resistance mutations have not developed the R263K mutation when they were subsequently treated with DTG and failed therapy, suggesting that RAL- and EVG-specific resistance mutations may be

incompatible with the development of R263K. To confirm this hypothesis, we performed tissue culture selection experiments with DTG using RAL/EVG resistant viruses and showed that the presence of either the E92Q or N155H substitutions was compatible with the emergence of R263K whereas the G140S/Q148R, E92Q/N155H, G140S and Q148R mutations were not. We assessed the effects of combining R263K with primary RAL and EVG resistance mutations on both integrase activity and HIV-1 replicative capacity in the presence and absence of INSTIs and showed that the N155H/R263K combination did not further decrease HIV-1 infectivity but increased resistance to DTG compared to R263K alone, suggesting this combination may be relevant to patients receiving DTG as a second line regimen. This work provides further confirmation of the uniqueness of the R263K resistance pathway and helps to explain its absence in a second line therapeutic setting.

3.1.3 Author Summary

Three integrase strand transfer inhibitors are currently available to individuals living with HIV: raltegravir, elvitegravir and dolutegravir. In contrast to other drugs, dolutegravir has not selected for resistance in HIV-positive individuals when used in first line therapy. Our group has previously selected a R263K dolutegravir resistance mutation in HIV through tissue culture selection experiments. The current study aimed to uncover whether mutations that confer resistance against raltegravir and elvitegravir were compatible with R263K. We found that only one resistance mutation, N155H, increased both viral fitness and levels of resistance against dolutegravir when associated with R263K compared to R263K alone, and this combination also showed increased resistance to elvitegravir compared to N155H alone. This suggests that this pathway may be able to occur among

elvitegravir failures who later receive dolutegravir in therapy. However, the emergence of R263K is not followed by that of N155H, E92Q, or any other mutation in the clinic, suggesting that E92Q and N155H can precede but not follow the occurrence of R263K.

3.2 Introduction

Although highly active antiretroviral therapy (HAART) has dramatically changed the outlook of patients diagnosed with HIV, resistance has emerged for every class of drugs (88). Integrase strand transfer inhibitors (INSTIs) have been an important addition to the arsenal of antiretroviral drugs and proper function of the viral integrase protein (IN) is essential to HIV integration and productive infection. Integrase catalyzes two reactions: 3' processing of linear, reverse-transcribed viral DNA (vDNA) and its insertion into the host genome, an event termed strand transfer (222). INSTIs specifically inhibit this second step but both of the first INSTIs, raltegravir (RAL) and elvitegravir (EVG), are susceptible to virological failure due to primary resistance mutations that increase resistance at the expense of fitness and secondary resistance mutations that develop after primary mutations and compensate for the decrease in fitness and/or further increase resistance (115). In contrast, the newer INSTI dolutegravir (DTG) has yet to select a major resistance mutation when used in first-line therapy (223). Our group has previously been able to select the R263K substitution in HIV-1 IN through passage of subtype B virus in cord blood mononuclear cells (CBMCs) in increasing concentrations of DTG (135). We have also previously shown that this mutation confers moderate resistance to DTG and EVG, while also decreasing the activity of IN by ~30% compared to wild-type (169). Results from the SAILING clinical trial, in which INSTI-naive treatment-

experienced participants received either DTG or RAL, support our observations (113). Indeed, although DTG was shown to be superior to RAL in this study and did not select for any classical INSTI resistance mutations, two patients who received DTG acquired the R263K mutation, which was associated with less than a 2-fold change in resistance and these two patients remained on DTG therapy and continued to perform well (113).

Some individuals who had previously failed RAL-based regimens and who possessed resistance mutations at position 148 were shown to subsequently fail DTG-based therapy in the VIKING trial, and the likelihood of failure increased with the number of additional secondary INSTI resistance mutations (145). However, none of the participants developed the R263K resistance mutation during virological failure, suggesting that the mutations that confer primary resistance to RAL/EVG might be incompatible with the R263K substitution. We sought to determine whether viruses bearing primary resistance mutations against RAL and/or EVG could select for resistance against DTG through acquisition of R263K and what the effect of the combination of R263K together with the primary INSTI resistance mutations E92Q, Y143R, Q148R, and N155H might be on both integrase activity and HIV-1 infectivity. Here, we demonstrate that only the combination of R263K with the N155H substitution led to an increase in both infectivity and DTG resistance compared to R263K alone, while the combination of R263K with any other primary INSTI resistance mutation resulted in failure of viruses to replicate or did not result in any additional increase in infectivity or drug resistance compared with the presence of R263K alone. Consistent with this, the selection of R263K was possible when a N155H-harbouring virus was subjected to DTG pressure. This suggests that a new pathway through which resistance to dolutegravir may ultimately develop *in vivo* may

be possible. However, the presence of R263K prior to N155H never led to the subsequent selection of the latter mutation. This is consistent with the hypothesis that the development of R263K may in fact be beneficial to a patient, as it traps the virus in an evolutionary dead end from which it is unable to develop additional compensatory mutations. In this circumstance, the fitness of HIV cannot be restored and moderate to high levels of DTG resistance are unable to emerge.

3.3 Materials and Methods

3.3.1 Cells and reagents

TZM-bl and 293T cells were cultured as in Chapter 2 Section 2.3.2. Cord blood mononuclear cells were obtained from the Department of Obstetrics, Jewish General Hospital, Montréal, Canada and were isolated by Ficoll-Hypaque (GE Healthcare) gradient centrifugation (224). Merck & Co., Inc., Gilead Sciences, Inc. and ViiV Healthcare Ltd. supplied raltegravir (RAL), elvitegravir (EVG) and dolutegravir (DTG), respectively. Compounds were solubilised in dimethyl sulfoxide.

3.3.2 Selection in cord blood mononuclear cells

Infection of CBMCs with $NL4.3_{wt}$, $NL4.3_{IN(E92Q)}$, $NL4.3_{IN(N155H)}$, $NL4.3_{IN(E92Q/N155H)}$, $NL4.3_{IN(Q148R)}$, $NL4.3_{IN(G140S)}$, and $NL4.3_{IN(G140S/Q148R)}$ viruses in increasing concentrations of DTG was performed as reported previously in (191, 225). DNA mutations were identified through sequencing of the integrase region of the HIV-1 *pol* gene as described in (226).

3.3.3 Integrase strand-transfer activity assay

Site-directed mutagenesis was performed as described Chapter 2 Section 2.3.7 to introduce the primary mutations E92Q, Y143R, Q148R, and N155H into the pET15b$_{wt}$ and pET15b$_{R263K}$ integrase subtype B expression vectors. See Table 3.1 for sense and antisense primer sequences. The generation of the construct bearing the R263K mutation has already been described (135). Recombinant integrase proteins were expressed in BL21 (DE3) bacterial strain cells and purified as described in Chapter 2 Section 2.3.11. Strand-transfer assays were performed using pre-processed HIV-1 LTR DNA as follows: DNA Bind 96-well plates (Corning) were coated with 50 µM LTR DNA for 48h at 4°C and then incubated with 600 nM purified protein. Afterwards, 1:2 serial dilutions of biotinylated target DNA was added and plates were incubated at 37°C for 1h to allow the strand-transfer reaction to occur. Integrated target DNA was quantified through the use of europium (Eu)-labelled streptavidin, which stably bind the biotin tag of the target DNA molecules (135).

Integrase mutation	5' to 3' primer sequence
E92Q	Sense:GCAGAAGTAATTCCAGCACAGACAGGGCAAGAAA Antisense:TTTCTTGCCCTGTCTGTGCTGGAATTACTTCTGC
G140S	Sense:GGGGATCAAGCAGGAATTTAGCATTCCCTACAATC Antisense:GATTGTAGGGAATGCTAAATTCCTGCTTGATCCCC
Y143R	Sense:CAGGAATTTGGCATTCCCCGCAATCCCCAAAGTCAGGG Antisense:CCCTGACTTTGGGGATTGCGGGGAATGCCAAATTCCTG
Q148R	Sense:ATTTGGCATTCCCTACAATCCCCAAAGTAGAGGAGTAATAGAATCTATG Antisense:CATAGATTCTATTACTCCTCTACTTTGGGGATTGTAGGGAATGCCAAAT
N155H	Sense: AAAGTCAGGGGGTAATAGAATCTATGCATAAAGAATTAAAGAAAATTATAGGAC Antisense: GTCCTATAATTTTCTTTAATTCTTTATGCATAGATTCTATTACCCCCTGACTTT

Table 3.1: Sequences of primers used in this study. Primer sequences used to introduce amino acid substitutions in HIV-1 integrase in both the pNL4.3 and pET15b vectors.

3.3.4 Generation of NL4.3 HIV-1 viral clones

The generation of the pNL4.3$_{IN(R263K)}$ plasmid has been reported previously in Chapter 2 Section 2.3.7; Similar methods were used to generate the pNL4.3$_{IN(E92Q)}$, pNL4.3$_{IN(Y143R)}$,

pNL4.3$_{IN(G140S)}$, pNL4.3$_{IN(Q148R)}$, pNL4.3$_{IN(N155H)}$, pNL4.3$_{IN(E92Q/N155H)}$, pNL4.3$_{IN(E92Q/R263K)}$, pNL4.3$_{IN(Y143R/R263K)}$, pNL4.3$_{IN(G140S/Q148R)}$, pNL4.3$_{IN(Q148R/R263K)}$, pNL4.3$_{IN(N155H/R263K)}$ plasmids through site-directed mutagenesis using the primers listed in Table 3.1. Genetically homogenous viral stocks were produced as described in Chapter 2 Section 2.3.7. Briefly, 293T cells were transfected with the various pNL4.3 plasmids using Lipofectamine 2000. At 48h after transfection, cell culture supernatants were collected and filtered at 0.45µm to remove plasmids and cell debris. Viruses were then aliquoted and stored at -80°C. Viral stocks were quantified by measuring cell-free reverse transcriptase activity.

3.3.5 HIV susceptibility to integrase strand-transfer inhibitors

Infectivity assays were performed as described in Chapter 2 Section 2.3.8. HIV susceptibilities to DTG, RAL and EVG were measured by the infection of 30,000 TZM-bl cells using 200,000 RT units per well of each virus in the presence of 1:4 serial dilutions of drugs. After 48h, cells were lysed and luciferase production was measured using the Luciferase Assay System (Promega, Madison, WI, Canada).

3.3.6 HIV infectivity and replication capacity

HIV-1 infectivity was measured through the infection of 30,000 TZM-bl cells per well using serial 1:4 dilutions of the NL4.3 viral clones. Levels of infection were measured as described above.

3.3.7 Statistical analysis

Each experiment is an average of at least two biological replicates performed in triplicate ($n \geq 6$). Biochemical assays of strand-transfer activity were normalized to wild-type activity at 128nM target DNA (Fig 3.1 and Table 3.2). Relative EC_{50} was normalized to wild-type

EC_{50} (Table 3.3). Fold change (FC) was normalized to wild-type K_M (Table 3.2), wild-type K_i (Fig 3.2) or wild-type IC_{50} (Table 3.4). K_M, V_{max}, K_i, EC_{50}, IC_{50}, standard error of the mean (SEM), one-way ANOVA, and 95% confidence intervals were calculated using Prism 5.0 Software. Fig 3.1 and Fig 3.2 were produced using Prism 5.0 Software.

3.4 Results

3.4.1 The addition of R263K to classical INSTI resistance mutations negatively impacts integrase strand-transfer efficiency

First, we performed biochemical analyses of the strand-transfer efficiencies of integrase proteins containing the E92Q, Y143R, Q148R and N155H primary INSTI resistance substitutions, both alone and in combination with the R263K substitution (Fig 3.1). Table 2 summarizes the enzyme kinetic values, K_M and V_{max}, that were derived from the substrate curves presented in Fig 3.1 with 95% confidence intervals and fold change (FC) for K_M. These results show that each single mutation had a negative impact on the strand-transfer activity of integrase, in accordance with previous findings, but that this effect was exacerbated when these substitutions were combined with R263K. This is also clear from lower maximal activities and shallower curves in Fig 3.1, and lower V_{max} and higher K_M values in Table 3.2 as seen with the double mutants compared to either single mutants or wild-type. Amongst single mutant proteins, the Q148R- and N155H- containing integrases were the most impacted with regard to K_M, with fold changes of 2.49 and 2.72 from wild-type, respectively. Combinations of E92Q/R263K and Q148R/R263K resulted in the lowest levels of strand-transfer activity (Fig 3.1, Table 3.2).

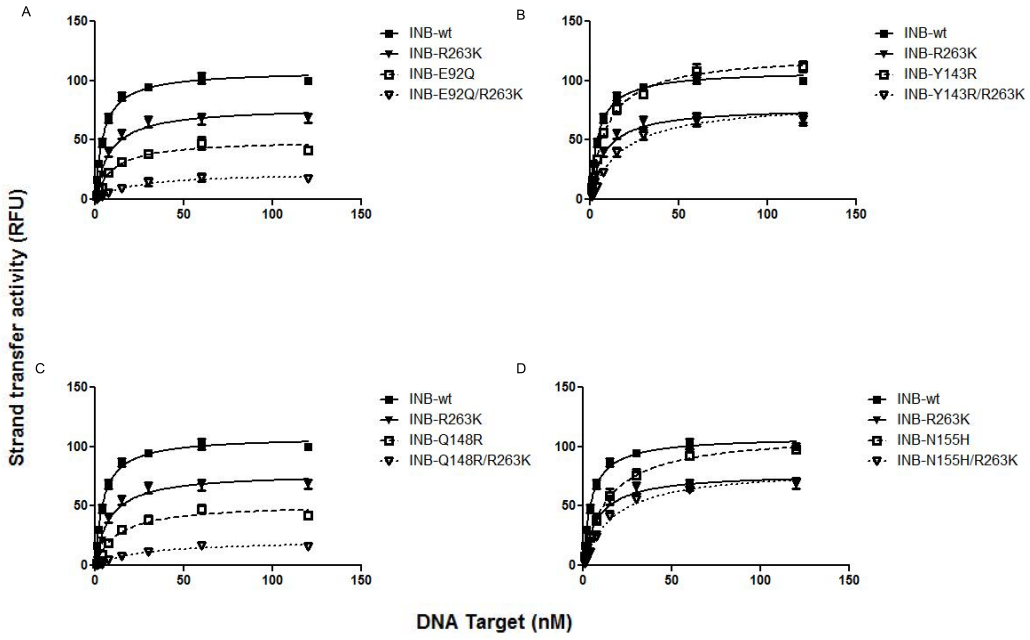

Figure 3.1: Relative strand transfer efficiencies of mutant integrase proteins when varying biotinylated target DNA concentrations. Wild-type and R263K integrase proteins are included as internal controls in each panel: A) E92Q ± R263K, B) Y143R ± R263K, C) Q148R ± R263K and D) N155H ± R263K. Error bars show standard error of the mean (SEM). The addition of R263K to each single resistance mutation led to a decrease in strand transfer activity in each case.

	Cell-free strand transfer assays			
Genotype	K_m (nM)	95% CI	FC	V_{max}
WT	5.07	4.30–5.85	1.00	100.00
E92Q	9.67	4.64–14.69	1.91*	46.27
Y143R	9.39	7.81–10.98	1.85*	112.88
Q148R	12.60	5.17–20.03	2.49*	48.07
N155H	13.78	12.07–15.50	2.72*	102.87
R263K	9.94	7.48–12.39	1.96*	68.72
E92Q R263K	21.23	9.68–32.78	4.19*†	21.08
Y143R R263K	18.03	10.63–25.43	3.56*†	76.69
Q148R R263K	26.64	8.83–44.46	5.26*†	19.70
N155H R263K	16.99	11.50–22.47	3.35*†	76.14

Table 3.2: Biochemical characterization of strand transfer activity of wild-type and mutant recombinant integrase proteins. Shown are the effects of primary integrase strand transfer inhibitor (INSTI) resistance mutations ± R263K on the K_M and V_{max} (relative to the WT) in cell-free assays. The fold-change (FC) for K_m and the 95% confidence intervals (95% CI) are reported. *, $p \leq 0.05$ for Student's *t*-test comparing K_M to the WT value; †, $p \leq 0.05$ for Student's *t*-test comparing K_M to the R263K mutant value.

3.4.2 The addition of R263K to classical INSTI resistance mutations increases resistance to DTG

Since the emergence of HIV resistance mutations relies on changes in both viral fitness and levels of resistance, we next investigated the impact of these different mutations on integrase susceptibility to DTG, as measured by the fold change in the inhibitory constant K_i for DTG of each purified recombinant integrase protein in cell-free strand-transfer assays (Fig 3.2). The data show that enzymes bearing the R263K or Y143R unique mutations displayed an approximate 2-fold increase in resistance compared to wild-type. All integrase proteins containing RAL/EVG resistance substitutions at positions 92, 143, 148, and 155 with R263K were less susceptible to DTG than wild-type enzyme. Furthermore, the addition of R263K to E92Q, Q148R, and to a lesser degree to N155H, decreased integrase susceptibility to DTG compared to the single E92Q, Q148R and N155H mutants, respectively. In contrast, no increase in resistance was observed when the R263K substitution was added to Y143R (2.1-fold change for both the single and double mutants). While increases in resistance were moderate for the E92Q/R263K and N155H/R263K combinations, the Q148R/R263K combination conferred 3.9 and 2.2 times higher resistance than was seen with enzymes containing Q148R and R263K alone.

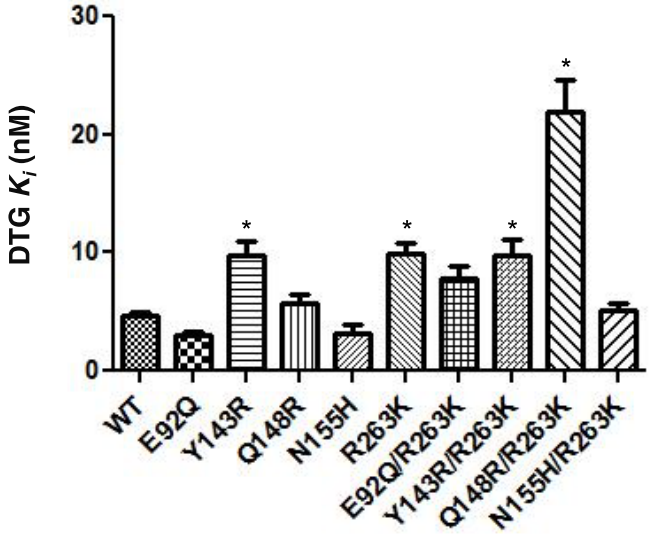

Figure 3.2: Inhibitory constants (K_i) for dolutegravir (DTG) measured in a cell-free system for recombinant integrase B proteins. Error bars indicate standard error of the mean (SEM). * indicates $p \leq 0.05$ for Student's *t*-test comparing each column to WT. Only the combination of Q148R/R263K led to an increase in DTG resistance compared to the R263K single mutant.

3.4.3 The addition of N155H to R263K partially restores viral infectivity

Given that cell-free assays do not always reproduce cell-based results, we next measured the effects of these mutations in isolation or in conjunction with R263K on HIV-1 relative infectivity, with 95% confidence intervals reported (Table 3.3). In agreement with the biochemical data (Fig 3.1 and Table 3.2), each substitution in isolation had a negative impact on HIV-1 infectivity, when compared to WT. The $NL4.3_{IN(Y143R/R263K)}$ and $NL4.3_{IN(Q148R/R263K)}$ viruses showed the greatest decline in infectivity with increases in relative EC_{50} of 17.9- and 29.6-fold compared to wild-type, respectively. Importantly, the $NL4.3_{IN(N155H/R263K)}$ virus had a lower EC_{50} than did the $NL4.3_{IN(R263K)}$ single mutant (FC=1.2 for N155H/R263K compared to 2.7 for R263K alone), suggesting that the addition of N155H partially restored the deficit in infectivity observed with R263K.

Genotype	Relative EC_{50}	95% CI
WT	1.00	0.67–1.48
E92Q	1.46†	1.08–1.96
Y143R	1.28†	0.97–1.69
Q148R	6.06*†	4.84–7.59
N155H	1.27†	0.77–2.08
R263K	2.66*	1.90–3.73
E92Q R263K	3.78*	2.72–5.26
Y143R R263K	17.98*†	11.61–27.60
Q148R R263K	29.61*†	8.53–102.83
N155H R263K	1.19†	0.86–1.64

Table 3.3: Infectivity of wild-type and mutant NL4.3 clones in TZM-bl cells. Shown are the effects of primary integrase strand transfer inhibitor (INSTI) resistance mutations ± R263K on the EC_{50} (relative to WT) of NL4.3 virus in TZM-bl cells. The 95% confidence intervals (95% CI) are reported. *, $p \leq 0.05$ for Student's *t*-test comparing each value to the WT value; †, *, $p \leq 0.05$ for Student's *t*-test comparing each value to the R263K mutant value.

3.4.4 The addition of R263K to classical INSTI resistance mutations does not affect resistance to RAL but increases resistance to both DTG and EVG

We next measured HIV-1 susceptibility to DTG, RAL and EVG in tissue culture through the determination of 50% inhibitory concentration (IC_{50}) values for viruses bearing primary INSTI resistance mutations alone or in combination with R263K (Table 3.4). FC and 95% confidence intervals are shown for each virus studied. In agreement with previous work (162, 227), single mutants with the exception of R263K did not confer significant levels of resistance against DTG, i.e. the IC_{50}s of all viruses harboring unique classical resistance mutations were at or below the level of wild-type. The $NL4.3_{IN(R263K)}$ virus displayed a 2-fold increase in resistance compared to WT in agreement with previous studies (135, 168, 169, 228). As mentioned above, the impairment in replication seen in the $NL4.3_{IN(Y143R/R263K)}$ and $NL4.3_{IN(Q148R/R263K)}$ viruses impeded data collection. Similar to the cell-free assay (Fig 3.1), the addition of R263K to $NL4.3_{IN(E92Q)}$ increased the IC_{50} for DTG to 24.9 nM compared to 18.2 nM for WT (1.4-fold), whereas the IC_{50}s were 13.2 nM and

36.3 nM for NL4.3$_{IN(E92Q)}$ and NL4.3$_{IN(R263K)}$, respectively. Importantly, the addition of N155H to R263K, which partially restored the defect in infectiousness associated with the latter mutation, also increased resistance against DTG (from 2.0 to 3.3 fold compared to WT).

The addition of R263K to the classical INSTI resistance mutations did not appear to have a major effect on HIV-1 susceptibility to RAL. Of the double mutants that we were able to assay, only minor changes in IC_{50} values were observed. Both the NL4.3$_{IN(E92Q/R263K)}$ and NL4.3$_{IN(N155H/R263K)}$ viruses were associated with slight decreases theIC_{50}s for RAL compared to the single mutant viruses, i.e. from 2.8 nM to 2.4 nM and from 6.1 nM to 3.7 nM, respectively. With respect to EVG, the NL4.3$_{IN(E92Q)}$ virus was already quite resistant to this drug and was unaffected by the additional presence of R263K. In contrast, the addition of R263K to N155H increased resistance to EVG by 1.4-fold.

	DTG			RAL			EVG		
Genotype	IC_{50} (nM)	95% CI	FC	IC_{50} (nM)	95% CI	FC	IC_{50} (nM)	95% CI	FC
WT	18.17	15.04–21.93	1.00	114.50	69.69–188.1	1.00	136.9	51.81–361.7	1.00
E92Q	13.20	9.52–18.28	0.73†	325.20	124.8–847.2	2.84†	>5,000		>50*†
Y143R	19.65	15.41–25.06	1.08†	1,173.00	321.6–4,276.0	10.24*†	282	39.27–2,022	2.06†
Q148R	2.33	1.54–3.53	0.13*†	>5,000		>50*†	>5,000		>50*†
N155H	1.81	1.31–2.50	0.10*†	695.80	566.0–855.4	6.08*†	646	387.9–1,076	4.72*†
R263K	36.28	35.24–37.36	2.00*	55.45	32.99–93.19	0.48	2,539	1,819–3,544	18.55*
E92Q R263K	24.86	22.92–26.96	1.37*†	277.60	134.8–571.7	2.42†	>5,000		>50*†
N155H R263K	60.28	29.22–124.4	3.32*	421.90	293.1–607.3	3.68*†	889	439.7–1799	6.49*†
Y143R R263K		No data			No data			No data	
Q148R R263K		No data			No data			No data	

Table 3.4: Determination of IC_{50} values for wild-type and mutant NL4.3 viruses for DTG, RAL, and EVG. Shown are the effects of primary integrase strand transfer inhibitor (INSTI) resistance mutations ± R263K on IC_{50}s in TZM-bl cells for dolutegravir (DTG), raltegravir (RAL), and elvitegravir (EVG). The 95% confidence intervals (95% CI) and fold change (FC) are reported.*, $p \leq 0.05$ for Student's *t*-test comparing each IC_{50} to the WT value; †, $p \leq 0.05$ for Student's *t*-test comparing each IC_{50} to the R263K mutant value.

3.4.5 The E92Q and N155H viruses are able to select for the R263K mutation in tissue culture selection experiments but R263K containing viruses do not select for either E92Q or N155H

Since the combinations of E92Q and especially N155H together with R263K led to increased DTG resistance in tissue culture, compared to results for each mutation alone, we investigated whether viruses containing resistance mutations against RAL/EVG would permit the emergence of the R263K mutation during selection experiments in the presence of DTG. As seen in Table 3.5, the $NL4.3_{wt}$, $NL4.3_{IN(E92Q)}$, and $NL4.3_{IN(N155H)}$ viruses were all able to select for the R263K DTG-signature mutation under DTG pressure. In contrast, the E92Q/N155H combination of mutations did not lead to the emergence of any additional mutations, not even when low concentrations of DTG were used. Similarly, the Q148R substitution either in isolation or in combination with the G140S substitution, did not permit the selection of R263K. In contrast, the G140S/Q148R double mutant virus was able to select for H51Y, a secondary resistance mutation that has been associated with DTG resistance in culture in conjunction with R263K (169).

Starting genotype	Drug concn (μM)	New mutation(s) selected
WT	0.03	R263K
E92Q	0.05	R263K
N155H	0.10	R263K
E92Q N155H	0.05	None
Q148R	0.25	None[b]
G140S[c]	0.50	V13I, V54I, Q148R
G140S Q148R	0.50	K14R, H51Y/H, V54I

Table 3.5: Emerging mutations in integrase detected by genotyping viruses containing INSTI resistance-associated mutations that were grown under DTG pressure for 30 weeks. Shown are changes in the integrase-coding sequence from HIV-1 subtype B NL4.3 clonal viruses during a 30-week dolutegravir (DTG) selection. The highest drug concentrations reached with each virus are noted.
[b] Reversion to Q148.
[c] G140S is a common secondary mutation to Q148R.

3.5 Discussion

Integration of HIV-1 vDNA into the host cell genome is essential in the lifecycle of the virus, making this step an attractive target for drug development. Early INSTIs have seen much success in the clinic (reviewed in (229, 230)); however their efficacies have been limited by the development of drug resistance (231). DTG has the potential to greatly impact the AIDS epidemic as it has a higher genetic barrier to resistance than RAL or EVG but the selection of the R263K mutation in both tissue culture and in the clinic in treatment-experienced individuals raises concerns that the development of resistance against this new compound may only be a matter of time (135). Importantly, no such resistance mutation has been observed when DTG has been used in first line therapy and more research into the resistance profile of DTG needs to be undertaken. We believe that selection of R263K in INSTI-naive individuals may represent an evolutionary dead-end for HIV from which it may not be able to escape. This hypothesis is consistent with clinical data on the failure of DTG resistance to emerge in first line therapy. The selection of the R263K mutation may well be beneficial for patients who are infected by HIV.

This is the first study to evaluate the combination of the DTG mutation R263K together with primary resistance mutations that arise from RAL or EVG drug pressure. We have shown in both cell-free and tissue culture assays that the addition of R263K to classical INSTI resistance mutations almost always leads to an overall decrease in integrase enzyme efficiency and viral infectivity (Fig 3.1, Tables 3.2 and 3.3) and with the exceptions of Q148R/R263K in cell-free assays (Fig 3.2) and N155H/R263K in tissue culture (Table 3.4), these combinations did not lead to increased resistance to DTG. Viruses containing Q148R and R263K together have never been reported in the clinic,

consistent with the fact that this combination results in a severe impairment in both integrase activity and viral infectivity (Tables 3.2 and 3.3). We therefore hypothesize that the negative effect of this mutational combination on the replicative capacity of the virus outweighs any potential benefit relative to enhanced resistance to DTG. Even though we have shown that viruses containing E92Q can select for R263K, we do not believe that this combination will be clinically relevant. The NL4.3$_{IN(E92Q/R263K)}$ clone showed a decrease in both infectivity and DTG resistance compared to NL4.3$_{IN(R263K)}$ alone, which suggests that the E92Q/R263K pathway is an inefficient route to resistance (Tables 3.3 and 3.4). Altogether, our results help to explain the absence of the R263K mutation in participants in the VIKING clinical trial who failed DTG after having previously failed either RAL or EVG with detectable resistance mutations (145).

Our results also suggest that the combination of N155H and R263K may be a novel resistance pathway. We were able to select R263K in combination with N155H when the NL4.3$_{IN(N155H)}$ virus was grown under DTG pressure (Table 3.5) and this combination positively impacted both HIV infectivity and DTG IC_{50}, relative to either single mutant (Tables 3.3 and 3.4). Based on these findings alone, we might have expected to see the emergence of N155H after R263K in DTG-treated patients, but this was not the case. This may be due, in part, to the extremely small number of patients who have failed DTG therapy as well as to the antiviral efficacy of this drug and its relatively recent clinical approval. However, the failure to select high level resistance to DTG in tissue culture and the failure to identify any compensatory mutation for R263K suggests that there is more to this issue. Because the N155H/R263K combination did result in a deficit in strand transfer when the recombinant protein was used in cell free assays (Fig 3.1 and Table

3.2) and only led to a modest increase in DTG resistance in tissue culture (~1.7 fold increase compared with R263K alone) and none with purified recombinant integrase (Fig 3.2 and Table 3.4), it is unclear how much of a selective advantage would be represented by this combination *in vivo.* Because the addition of R263K changes the NL4.3$_{IN(N155H)}$ virus from hyper-sensitive to moderately resistant to DTG, its selection in this background in tissue culture is not surprising. It is possible, however, that the small benefit provided by the reverse scenario, i.e. the selection of N155H in R263K-containing HIV, may be outweighed by the still potent inhibitory effect of the drug as well as the defect in replication of the R263K-harboring virus and the negative impact that this combination has on the enzymatic function of integrase. Consistent with the negative impact of this combination on the virus, passage of NL4.3$_{IN(R263K)}$ in the absence of DTG did not result in the selection of additional mutations, including N155H (data not shown). Further studies with non-human primate models (232), will provide further information on the ability of HIV to adapt to DTG pressure and possibly to develop drug resistance. Current studies in our laboratory are investigating the possibility of combining N155H/R263K with the most common secondary mutations associated with N155H in individuals failing RAL/EVG therapy. Since these secondary substitutions may positively impact N155H in terms of enzymatic activity, viral replicative capacity, and drug resistance, they may conceivably facilitate the emergence of the R263K substitution, thereby providing a mechanism through which high levels of DTG resistance might eventually develop.

This is the first reported instance of a genetic change in the HIV$_{IN(R263K)}$ virus that is able to restore the infectious deficit of this mutant. Previous studies with the secondary resistance mutations H51Y and E138K showed that these substitutions further increased

DTG resistance when studied in conjunction with R263K; however they also further decreased enzymatic activity and viral infectivity, and thus were not considered to have clinical relevance (169, 228). However, the combination of N155H and R263K is biologically relevant, as demonstrated by its detection in an individual failing treatment with RAL (233). Also, the N155H, but not R263K, substitution occurred concomitantly with the development of high level DTG resistance in a treatment-experienced patient who had first failed RAL (166), suggesting that both of these substitutions are important for EVG and DTG resistance. However, as of yet, no compensatory mutation for DTG has been observed during more than four years of tissue culture selection experiments following the emergence of R263K or in the clinic (113). One implication is therefore that N155H must appear before R263K in the rare individuals in whom these mutations are jointly observed, since N155H on its own does not confer HIV resistance against DTG (Fig 3.2 and Table 4). Therefore, the N155H/R263K combination of mutations may only ultimately be observed when RAL- or EVG- experienced patients are treated with DTG as a salvage therapy and when the N155H mutation is present at baseline before initiation of DTG treatment. As DTG is currently used as a salvage therapy for RAL and EVG, this scenario is not unlikely, and the development of high levels of INSTI cross-resistance is a real threat. This study shows that this phenomenon can develop through a hitherto unexplored combination of the primary mutation N155H with the DTG mutation R263K.

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Chapter 4

The sequential development of integrase strand transfer inhibitor resistance mutations

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4.1 Preface

In Chapter 3, we hypothesized that the combination of R263K with primary INSTI resistance mutations would lead to additive negative effects on both IN enzymatic activity and viral infectivity and were mostly correct. However, we did discover that the combination of N155H and R263K restored infectivity defects seen with either single mutant alone, while slightly enhancing DTG resistance *in vitro.* Since then, the N155H/R263K combination has been found in one patient failing first-generation INSTI therapy (177), and the N155H mutation has been increasingly associated with DTG failures of treatment-experienced patients in the clinic (137, 166). The N155H substitution is seen approximately 50% of the time with one other, additional secondary INSTI resistance substitution in patient-derived sequences (177). We therefore wondered whether any of these secondary substitutions could act to enhance viral replicative capacity and/or DTG resistance of our N155H/R263K double mutant virus. To this end,

we added each of the five most common N155H-associated secondary mutations to the N155H/R263K double mutant enzyme and viral expression constructs, and assayed biochemical activity, viral infectivity, and drug resistance of the triple mutants. The aim of this project was to investigate whether we could engineer a highly DTG-resistant virus that was replicatively competent.

4.1.2 Abstract

We have previously shown that the addition of the raltegravir/elvitegavir (RAL/EVG) primary resistance mutation N155H to the R263K dolutegravir (DTG) resistance mutation partially compensated for the fitness cost imposed by R263K while also slightly increasing DTG resistance *in vitro*. Since many patients failing RAL/EVG are given DTG as part of rescue therapy, and given that the N155H substitution is often found in combination with other compensatory resistance mutations in such individuals, we investigated the effects of multiple such substitutions within integrase (IN) on each of integrase function, HIV-1 infectivity, and levels of drug resistance. To this end, each of the L74M, E92Q, T97A, E157Q, and G163R substitutions were introduced into NL4.3 subtype B HIV-1 vectors harboring N155H and R263K in tandem. Relevant recombinant integrase enzymes were also expressed and purified and biochemical assays of strand-transfer efficiency as well as viral infectivity and drug resistance studies were performed. We found that the addition of T97A, E157Q, or G163R somewhat improved the affinity of $IN_{N155H/R263K}$ for its target DNA substrate while the presence of L74M or E92Q had a negative effect on this process. However, viral infectivity was significantly decreased from that of $NL4.3_{IN(N155H/R263K)}$ after the addition of each tertiary mutation and no increases in levels of DTG resistance were observed. This work shows that the compensatory mutations that evolve after N155H

under continued DTG or RAL/EVG pressure in patients are unable to improve either enzyme efficiency or viral infectivity in a N155H/R263K background.

4.1.3 Importance

In contrast to other drugs, dolutegravir has not selected for resistance in HIV-positive individuals when used in first line therapy. We had previously shown that HIV containing the primary raltegravir/elvitegravir resistance substitution N155H could select for R263K under dolutegravir pressure and that this virus was fit and displayed low-level resistance to dolutegravir. Therefore, the current study aimed to uncover whether accessory mutations that appear after N155H in response to raltegravir/elvitegravir were compatible with N155H and R263K. We found, however, that the addition of a third mutation negatively impacted both the enzyme and the virus in terms of activity and infectivity without large shifts in integrase inhibitor resistance. Thus, it is unlikely that these substitutions would be selected under dolutegravir pressure. These data support the hypothesis that primary resistance against DTG cannot evolve through RAL/EVG resistance pathways and that the selection of R263K leads HIV into an evolutionary dead-end.

4.2 Introduction

Integrase strand transfer inhibitors (INSTIs) constitute the newest class of drugs approved for the treatment of HIV. They act by inhibiting the HIV enzyme integrase (IN) at the essential step in the viral replication cycle of the insertion of viral DNA into the host cell genome (105). The older drugs of this class, i.e. raltegravir (RAL) and elvitegravir (EVG) can select for primary resistance mutations both in tissue culture and in the clinic, which

allows for the virus to escape drug pressure. Furthermore, there is a high degree of cross-resistance between these two drugs (130, 170, 229). In contrast, dolutegravir (DTG), a newer INSTI, appears to have a higher genetic barrier to resistance and is active against many but not all RAL and EVG resistant viruses, due to the fact that some of the RAL and EVG resistance mutations can also confer cross-resistance to DTG (162). However, DTG is able to select for a R263K substitution in IN, which yields a 3-4-fold level of resistance to this drug at the expense of ≈ 30% viral replicative capacity (169) and this has been reported both in tissue culture and in treatment-experienced, INSTI-naive patients (113, 135).

In the VIKING trial, INSTI-experienced patients with primary resistance mutations associated with RAL and EVG had lower success rates while on DTG than did patients who received DTG in first-line regimens, and resistance mutations at position 148 in IN were associated with the worst outcomes (145). There was, however, no selection of R263K in this trial, suggesting that RAL/EVG resistance substitutions might be incompatible with the R263K DTG resistance pathway. Indeed, we have previously shown that the combination of R263K with the most common primary RAL/EVG resistance substitutions resulted in decreased enzymatic activity of IN as well as overall diminished viral infectivity (159). The only combination that appeared to increase viral infectivity and resistance to DTG when compared to R263K was that of N155H together with R263K (10). However, the changes associated with the addition of N155H to R263K were small. Moreover, the N155H substitution in the context of RAL/EVG resistance is often associated with other secondary mutations in INSTI-experienced patients (233). Therefore, we evaluated whether the addition of some of these secondary substitutions

at positions L74M, E92Q, T97A, E157Q, and G163R to both N155H and R263K might influence IN enzymatic activity as well as viral infectivity and levels of INSTI drug resistance. These secondary substitutions were selected because they are the five most common to occur in patients failing INSTI-based therapy with the N155H primary substitution (233).

Although the addition of either T97A, E157Q, or G163R improved the affinity of IN for its DNA substrate, we found that none of the tertiary mutations that were tested could compensate for the decreased strand transfer activity of the double N155H/R263K mutant in biochemical assays. This finding was mirrored in tissue culture infections that assessed the infectivity of subtype B HIV-1 viruses harboring these same substitutions. In regard to resistance to INSTIs, all the triply mutated viruses were highly resistant to EVG while resistance against RAL and DTG was mostly unchanged in comparison to the effect of the N155H/R263K mutational combination.

4.3 Materials and Methods

4.3.1 Experimental design

The research objectives of this study were to evaluate the strand-transfer activity of recombinant integrase proteins expressing different combinations of point mutations that confer resistance to RAL and/or EVG and/or DTG. We also wished to assess the viral infectivity of equivalent NL4.3 viruses in the presence or absence of RAL, EVG, and DTG.

4.3.2 Cells and reagents

TZM-bl and 293T cells were cultured as reported previously in Chapter 2 Section 2.3.2 (135). Merck & Co., Inc., Gilead Sciences, Inc. and ViiV Healthcare Ltd. supplied raltegravir, elvitegravir, and dolutegravir, respectively.

4.3.3 Integrase strand-transfer activity assay

Site-directed mutagenesis was performed as described in Chapter 2 Section 2.3.7 to introduce the secondary mutations L74M, E92Q, T97A, E157Q, and G163R into the pET15b$_{N155H/R263K}$ integrase subtype B expression vectors (169). Sense and antisense primer sequences are listed in Table 4.1. The generation of the construct bearing the N155H/R263K mutations has already been described in Chapter 3 Section 3.3.3 (159). Recombinant integrase proteins were expressed in BL21 (DE3) bacterial cells and purified as published (135). Strand-transfer assays were performed using pre-processed HIV-1 LTR DNA as reported previously in Chapter 3 Section 3.3.3 (169). Briefly, DNA Bind 96-well plates (Corning) were coated with LTR DNA and then incubated with purified protein. Afterwards, biotinylated target DNA was added and plates were incubated at 37C for 1h to allow the strand-transfer reaction to occur. Integrated target DNA was quantified through the use of europium (Eu)-labelled streptavidin which stably binds to the biotin tag of the target DNA molecules.

4.3.4 Generation of NL4.3 HIV-1 viral clones

The generation of the pNL4.3$_{IN(N155H/R263K)}$ plasmid has been reported previously in Chapter 2 Section 2.3.7 (159). Similar methods were used to generate other pNL4.3IN$_{(mutant)}$ plasmids through site-directed mutagenesis using the primers listed in Table 4.1. Genetically homogenous viral stocks were produced as described previously (135). Briefly, 293T cells were transfected with the various pNL4.3 plasmids using

Lipofectamine 2000. At 48h after transfection, cell culture supernatants were collected and filtered at 0.45µm to remove plasmids and cell debris. Viruses were then aliquoted and stored at -80°C. Viral stocks were quantified by measuring cell-free reverse transcriptase (RT) activity in culture fluids.

Integrase mutation	5′ to 3′ primer sequence
L74 M	
Sense	TGGCTACATGAACTGCTACCATGATAACTTTTCCTTCTAAATG
Antisense	CATTTAGAAGGAAAAGTTATCATGGTAGCAGTTCATGTAGCCA
E92Q	
Sense	GCAGAAGTAATTCCAGCACAGACAGGGCAAGAAA
Antisense	TTTCTTGCCCTGTCTGTGCTGGAATTACTTCTGC
T97A	
Sense	AGCAGAGACAGGGCAAGAAGCAGCATACTTCCTC
Antisense	GAGGAAGTATGCTGCTTCTTGCCCTGTCTCTGCT
N155H/E157Q	
Sense	CCCAAAGTCAAGGAGTAATAGAATCTATGCATAAACAGTTAAA GAAAATTATAGGACAGGTAAGAGA
Antisense	TCTCTTACCTGTCCTATAATTTTCTTTAACTGTTTATGCATAGA TTCTATTACTCCTTGACTTTGGG
N155H/G163R	
Sense	GGAGTAATAGAATCTATGCATAAAGAATTAAAGAAAATTATAA GACAGGTAAGAGATCAGGC
Antisense	GCCTGATCTCTTACCTGTCTTATAATTTTCTTTAATTCTTTATG CATAGATTCTATTACTCC

Table 4.1: Primer sequences used to introduce mutations in HIV-1 integrase in both the pNL4.3 and pET15b vectors.

4.3.5 HIV susceptibility to integrase strand-transfer inhibitors

HIV susceptibilities to DTG, RAL and EVG were measured by the infection of 30,000 TZM-bl cells using 100,000 RT units per well of each virus in the presence of 1:10 serial dilutions of drugs. After 48h, cells were lysed and luciferase production was measured using the Luciferase Assay System (Promega, Madison, WI, Canada).

4.3.6 HIV infectivity and replication capacity

HIV-1 infectivity was measured through the infection of 30,000 TZM-bl cells per well using serial 1:4 dilutions of the various NL4.3 viral clones. Levels of infection were measured as described above.

4.3.7 Statistical analysis

Each experiment is an average of at least two replicates performed in triplicate (n=6). Biochemical assays of strand-transfer activity were normalized to wild-type (WT) activity at 1600nM protein (Fig. 4.1A) or 128nM target DNA (Fig. 4.1B). Relative infectivity index was normalized to wild-type EC_{50} (Table 4.2). Fold change (FC) was normalized to wild-type IC_{50} (Table 4.3). K_M, V_{max}, infectivity index, IC_{50}, standard error of the mean (SEM), and 95% confidence intervals were calculated using Prism 6.0 Software and all figures were visualized using this same software. Student's *t*-tests were performed using the OpenEpi toolkit, accessible free online at www.openepi.com.

4.4 Results

4.4.1 The effect of accessory mutations within $IN_{N155H/R263K}$ in biochemical assays

Figure 4.1A shows the strand transfer activities of all of the recombinant proteins that were studied as a function of protein concentration. All proteins displayed maximal activity at a concentration of 400 nM, except for E92Q/N155H/R263K which was maximally active at 200 nM. The N155H/R263K and N155H/G163R/R263K proteins displayed similar activities at both concentrations listed above. The N155H/R263K mutant was less active than WT at 400 nM, and this decrease was exacerbated by the addition of each of the tertiary mutations, especially L74M, E92Q, and G163R. All proteins lost activity at higher

concentrations, and this may be due to the formation of higher-order inactive integrase oligomers.

Figure 4.1B depicts the strand transfer activities of the recombinant proteins when target DNA concentration was varied. As the substrate of the enzyme increases, so does the activity of the protein until attainment of maximal activity, i.e. V_{max}. All mutants in this study exhibited both a lower V_{max}, and a shallower curve compared to WT; the E92Q/N155H/R263K-containing protein was especially inactive, while the other triple mutants appeared to perform in similar fashion to each other.

From the curves in Figure 4.1B, we derived V_{max} and K_M values, the latter being a measure of enzyme affinity for substrate. Figure 1C shows the changes in K_M upon the addition of each mutation. An increased K_M is indicative of a decrease in affinity for the target DNA. The results show that K_M was significantly increased ($p \geq 0.05$) upon the addition of any mutation when compared to WT. When compared to N155H/R263K, the addition of T97A, E157Q, or G163R were each able to significantly reduce the K_M of integrase, whereas the addition of L74M or E92Q were not. In fact, the E92Q/N155H/R263K-containing enzyme was so inactive that an accurate K_M could not be determined. When V_{max}/K_M which is a measure of enzyme proficiency was considered, however, we saw less of a difference among the various combinations of mutations. Although all decreases in enzyme efficiency were significant when compared to WT, the results of Fig 4.1D show that the addition of each tertiary mutation did not have a significant effect on the efficiency of the N155H/R263K-containing protein, although both the T97A/N155H/R263K and N155H/E157Q/R263K mutant proteins had increased V_{max}/K_M values. These results suggest that the addition of certain tertiary mutations to

integrase containing the N155H/R263K resistance mutations increased the affinity of the enzyme for its substrate but were generally unable to improve enzyme performance.

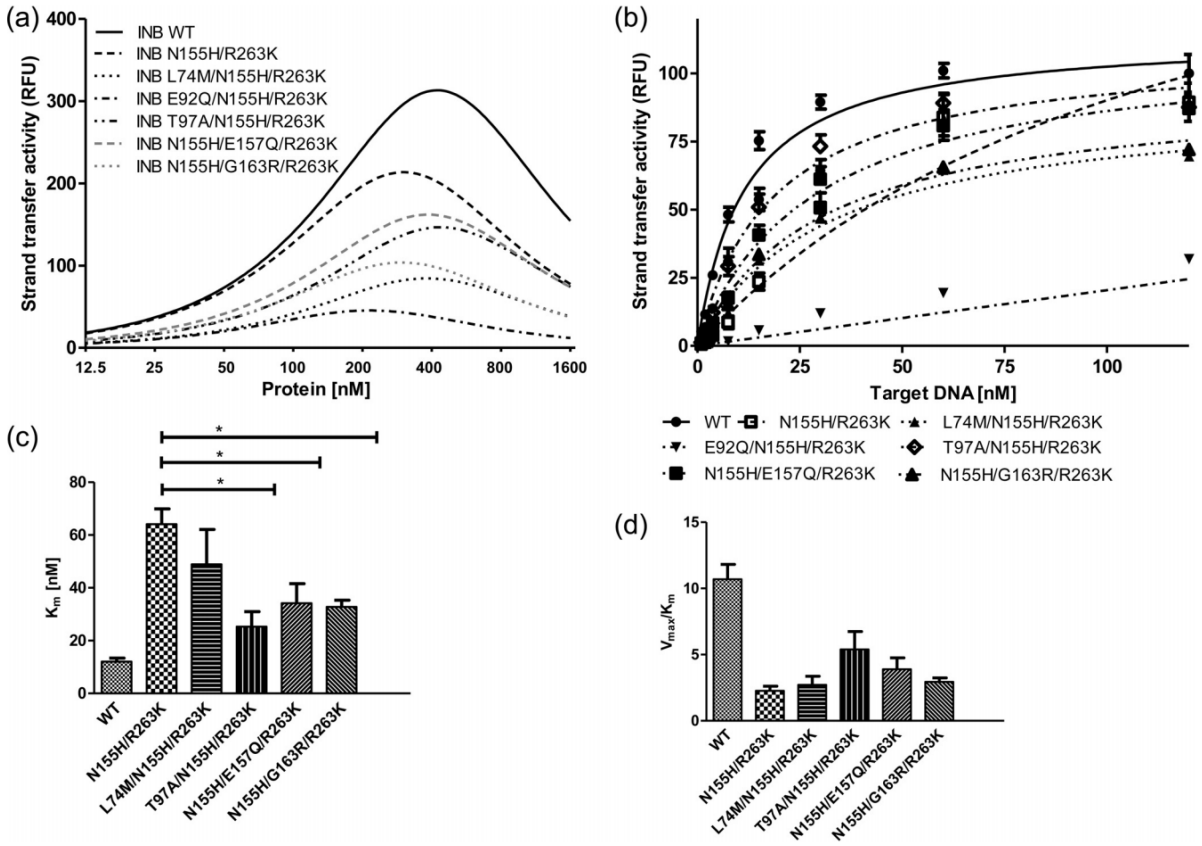

Figure 4.1: Strand transfer activities of purified recombinant integrase proteins in a biochemical assay. Biotinylated target DNA incorporation is measured by streptavidin fluorescence as a function of protein concentration (a) or target DNA concentration (b). (c) K_M values for each mutant were obtained from the slope of the curves shown in panel b. An asterisk indicates $p \leq 0.05$ by Student's *t*-test. (d) Enzyme proficiency is quantified by dividing the V_{max} (taken from the plateau in panel b) by the K_M. Error bars display SEM. The E92Q/N155H/R263K enzyme was too inactive to accurately permit calculation of K_M and was left out of panels c and d. RFU, relative fluorescence units.

4.4.2 The addition of accessory mutations to NL4.3$_{IN(N155H/R263K)}$ makes HIV-1 less infectious

Table 4.2 summarizes the results of experiments assessing the infectivity of NL4.3 viruses harbouring the N155H/R263K mutations, both alone and in combination with any additional tertiary resistance substitution that was studied. These values are derived from

the infectivity curves displayed in Figure 4.2 for each mutant. Similar to what was seen biochemically, the data show that the addition of each single mutation caused a significant decrease in infectivity (here represented as an increase in the infectivity index relative to WT) when comparisons were made to either WT or to the N155H/R263K double mutant. Thus, the decreased strand transfer activities described in Figure 4.1 correlate with a general negative effect on the analogous viruses in tissue culture. This trend can also be observed in Figure 4.2 that shows changes in luciferase reporter gene activity as a function of increasing concentrations of virus.

Genotype	Infectivity index[a]	95% CI
WT	1.00	0.86–1.17
N155H/R263K	1.60*	1.35–1.88
L74 M/N155H/R263K	4.04*†	3.51–4.66
E92Q/N155H/R263K	4.99*†	4.50–5.54
T97A/N155H/R263K	4.05*†	3.58–4.57
N155H/E157Q/R263K	3.75*†	3.38–4.16
N155H/G163R/R263K	2.73*†	2.25–3.32

Table 4.2: Effects of tertiary INSTI resistance mutations on the infectivity of HIV-1 in TZM-bl cells relative to that of the WT. Infectivity index and 95% confidence intervals (CI) are reported. An asterisk denotes an infectivity index that is statistically different from that of the WT by Student's *t*-test ($p \leq 0.05$). A dagger denotes an infectivity index that is statistically different from that of N155H/R263K by Student's *t*-test ($p \leq 0.05$).
[a] EC_{50} values relative to that of the WT.

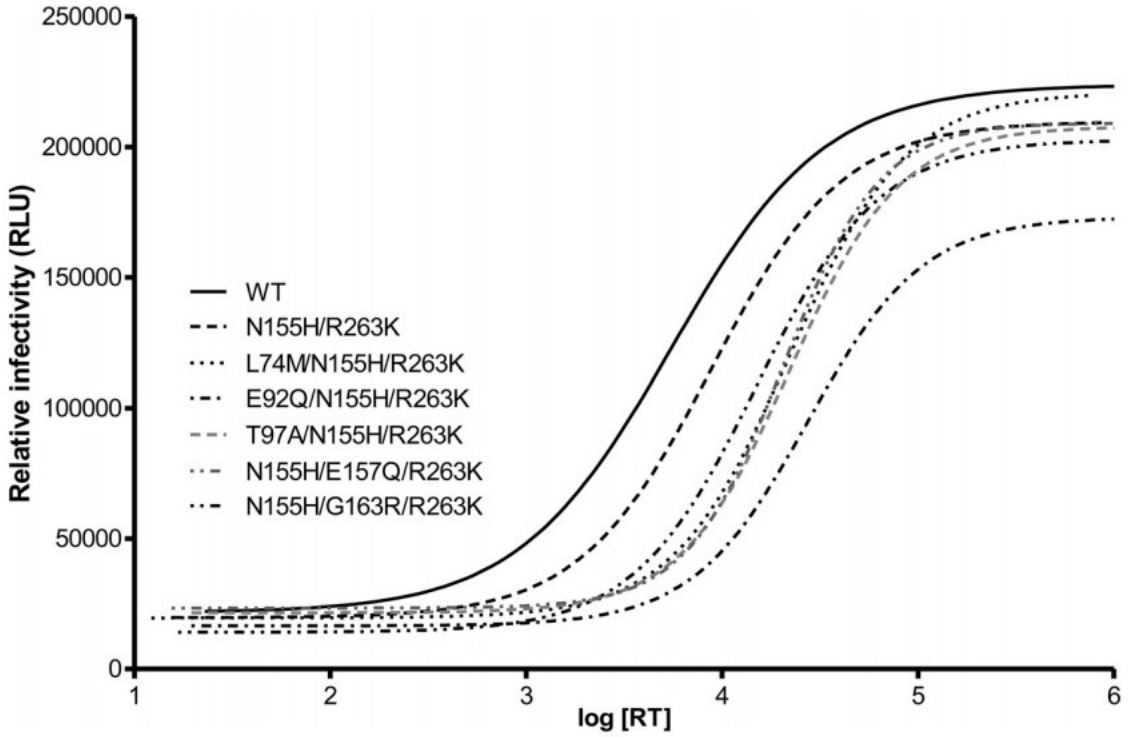

Figure 4.2: Infectivity in TZM-bl cells of WT, N155H/R263K, and triply mutated NL4.3 viruses. Relative infectivity measured by the luciferase fluorescence of cells 48 h after infection. RLU, relative luciferase units.

4.4.3 Accessory mutations have small effects on INSTI drug resistance in tissue culture

Figure 4.3 displays the results of resistance assays in tissue culture with the various drugs used in this study, i.e. (a) DTG, (b) RAL, and (c) EVG. When the concentration of drug was low, the negative effect of each mutation on viral replication was clearly seen. As drug concentration was increased, all of the mutants attained a growth advantage in the presence of drug and were able to replicate at higher drug levels than the WT virus. The IC_{50} values obtained in Figures 4.3A and B are summarized in Table 4.3. The addition of L74M to N155H/R263K virus increased HIV susceptibility to DTG by ~2-fold compared to $NL4.3_{IN(N155H/R263K)}$. Although resistance to DTG was relatively unchanged upon the addition of the other tertiary mutations, these same viruses displayed increased IC_{50}

values for RAL compared to either WT or the N155H/R263K double mutant, with the exception of the virus that contained G163R.

Similar findings are displayed in Figure 4.3C, with higher concentrations of EVG leading to a stepwise decline in infectivity of WT virus but not of the triple mutants, except at the highest concentration of EVG that was used. The calculation of IC_{50} values for EVG was not technically possible because very high levels of resistance against this drug had been reached. Although the WT virus exhibits a non-standard dose-response curve in Figure 4.3C, it is still evident that these mutants are all highly resistant as their infectivity in increasing concentrations of EVG is unaffected, except at the highest concentration tested. This points to an extremely high level of resistance to this drug in our assay for both the N155H/R263K double mutant, as well as all triply mutated viruses.

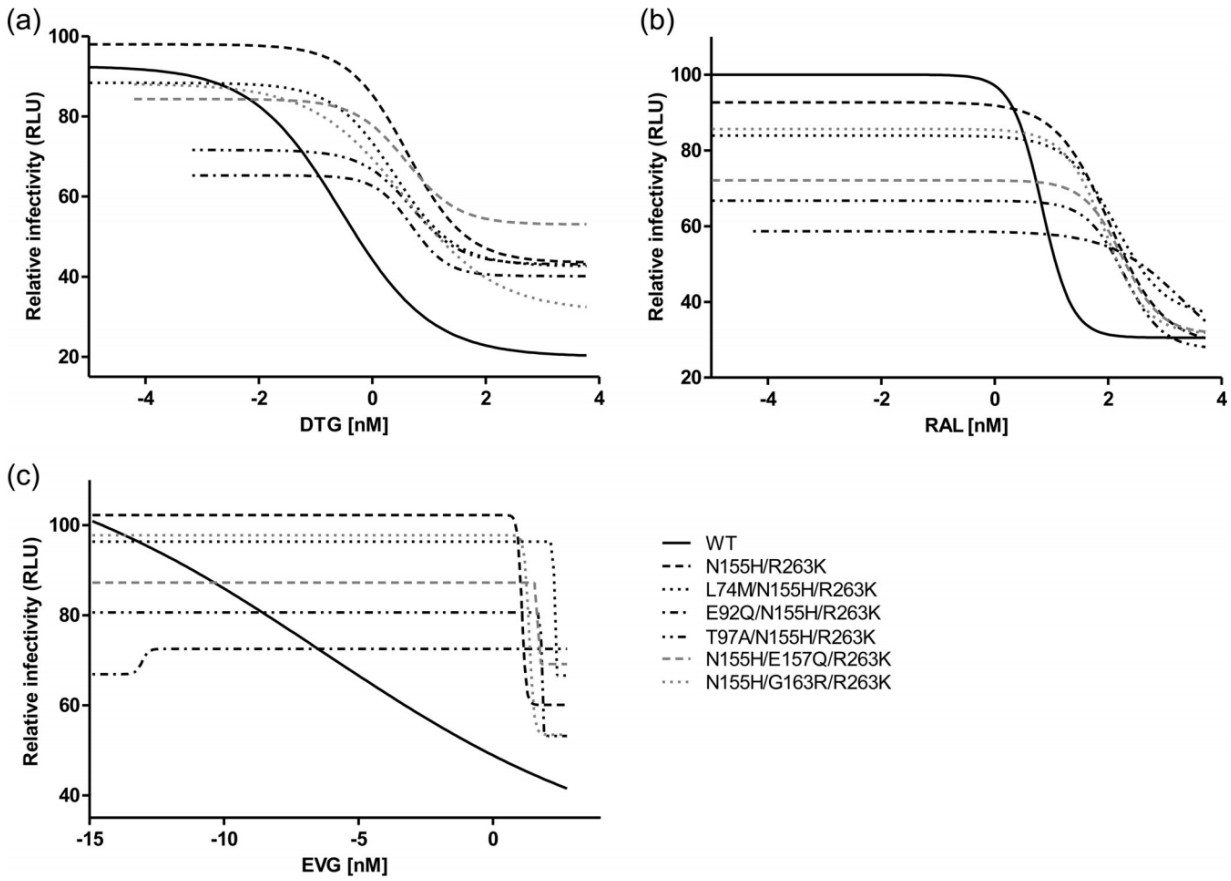

Figure 4.3: Infectivity of wild-type, N155H/R263K, and triply mutated NL4.3 HIV-1 in the presence of increasing concentrations of dolutegravir (DTG) (a), raltegravir (RAL) (b), and elvitegravir (EVG) (c).

	DTG			RAL		
Genotype	IC_{50} (nM)	95% CI	FC	IC_{50} (nM)	95% CI	FC
WT	0.284	0.164–0.494	1.00	6.797	5.580–8.279	1.00
N155H/R263K	4.202*	1.820–9.700	14.78	105.7*	59.76–186.8	15.55
L74 M/N155H/R263K	2.463*	1.167–5.199	8.66	136.9*	66.98–279.9	20.14
E92Q/N155H/R263K	4.922*	1.051–23.04	17.31	>340*		>50
T97A/N155H/R263K	4.889*	0.997–23.97	17.20	192.4*	117.2–315.9	28.31
N155H/E157Q/R263K	4.019*	0.897–18.02	14.14	177.7*	102.4–308.6	26.14
N155H/G163R/R263K	3.770*	1.431–9.930	13.26	88.83*	60.76–129.9	13.07

Table 4.3: Effects of tertiary INSTI resistance mutations on IC_{50}s in TZM-bl cells for DTG and RAL. 95% Confidence intervals (CI) and fold changes (FC) are shown. An asterisk denotes IC_{50}s significantly greater than that of the WT by Student's *t*-test ($p \leq 0.05$).

4.5 Discussion

Although combined antiretroviral therapy has revolutionized treatment for HIV-positive individuals, drug resistance has been observed for every class of drugs currently available

(88). DTG is a promising new INSTI that may defy this paradigm but only if it used in first-line therapy. In general, drug resistance is characterized by the initial presence of primary mutations that increase levels of resistance at the expense of viral replication capacity. Secondary mutations, that arise after primary mutations, are generally compensatory in nature and act to increase drug resistance while also restoring viral fitness (115). However, previous results have shown that the secondary mutations associated with DTG may counter-intuitively diminish viral fitness following the selection of a first mutation at position R263K.

Here, we investigated the effects of adding common INSTI secondary mutations to a N155H/R263K background that we had previously characterized as a relatively fit, DTG-resistant virus (159). We now show that the addition of any of five different tertiary substitutions had different effects on the strand transfer activity of IN. In our biochemical analyses, three mutations (T97A, E157Q, and G163R) improved the ability of IN to bind target DNA, but did not make a significant overall difference to enzymatic activity. Mirroring this, we saw a statistically significant negative effect on infectivity upon addition of each accessory mutation relative to both WT virus and to viruses containing N155H/R263K.

There are varying and sometimes conflicting reports on how these mutations act to increase fitness and/or drug resistance in response to RAL or EVG. One study showed that the addition of E92Q or G163R to N155H was able to increase viral fitness in both the absence and presence of RAL whereas the accessory mutation L74M only increased replication capacity in the presence of the drug (176). A different study showed that the E92Q substitution in combination with N155H was deleterious to HIV-1 replication in the

absence of INSTI pressure (164). The E157Q substitution has been shown to have variable effects on INSTI activity (187, 234) while a T97A substitution has been reported to cause a 70% decrease in viral replication relative to WT (164).

A recent study documented that viruses that contained the N155H mutation did not select for resistance to DTG via the acquisition of common compensatory substitutions; this finding is in agreement with our results. Interestingly, the selection of E92Q/N155H/R263K in response to EVG was reported in that study (154) and we show here that this virus is highly resistant to this INSTI.

It is generally agreed that the five accessory mutations discussed here contribute to higher levels of resistance to the first generation INSTIs, RAL and EVG, than do the primary mutations for these two drugs on their own (106, 164, 176, 235). In this study, we have expanded on these results by showing that each triply mutated virus is just as or more resistant to each INSTI than the N155H/R263K double mutant, with the exception of L74M/N155H/R263K for DTG and N155H/G163R/R263K for RAL. Most important, we have not observed significant changes in levels of resistance to DTG or RAL following the addition of any of these five compensatory mutations to backbone viruses. It appears that the presence of the R263K substitution that confers low-level resistance to DTG may prevent the development of further INSTI drug resistance substitutions and this is likely due to its effects on viral fitness. Investigations are underway in our laboratory to identify the precise mechanism in the context of the three-dimensional structure of IN through which the R263K substitution confers low-level resistance to DTG while preventing the generation of additional substitutions.

E157Q is particularly interesting because it is a naturally occurring polymorphism in HIV-1 subtype B (132), implying that it may be present in some patients at treatment initiation. E157Q is important because it confers some resistance to RAL and EVG on its own (235) and may thus facilitate the selection of further resistance to these drugs. Investigations in our laboratory are also underway to deduce whether this polymorphism has an effect on the emergence of R263K and resistance to DTG with both laboratory strains and viruses derived from INSTI-experienced patients bearing the E157Q substitution. A RAL-experienced patient who possessed numerous RAL-related mutations was recently reported to have failed DTG while also possessing E157Q (187). We investigated the substitution on its own and in combination with R263K and found that it restored the enzymatic defects of the IN_{R263K} while further increasing DTG resistance by 10-fold (161). Therefore, the combination of E157Q and R263K represents a pathway along which high levels of DTG resistance could develop.

A recent report from the P1093 DTG dose-ranging study identified a potentially non-adherent, treatment-experienced but INSTI-naive adolescent with low-level viremia and the emergence of the R263K substitution. In this case study, secondary resistance mutations were observed that modestly increased the level of DTG resistance. However, none of these substitutions were able to compensate for the decrease in replicative capacity conferred by R263K, consistent with the data presented here and with previous results (168, 169, 184, 228).

DTG is approved for use in both first and second line therapy. In initial therapy, DTG is unique in that no case of resistance against it or the nucleoside drugs with which it has been co-utilized has yet been reported (110, 213). However, when DTG is used as

a second line drug or in salvage therapy after prior failure on either RAL or EVG, it is far less effective due to cross-resistance against DTG that is conferred by RAL/EVG classical resistance mutations. The absence of resistance to DTG in initial therapy may be due to a relatively low level of resistance that is conferred by R263K coupled with a significant diminution in viral replication ability. We hypothesize that the N155H/R263K DTG resistant virus may not be able to develop additional resistance substitutions or to acquire increased fitness due to constraints on viral mutability and replication that are primarily imposed by the R263K mutation (10). Indeed, the N155H substitution has been associated with DTG failure in treatment-experienced patients (147, 166, 233, 236) including two INSTI-naive subjects recently reported in the SAILING trial. Although this is the first documentation of N155H in patients treated with DTG and not RAL, it is noteworthy that these two individuals possessed non subtype B viruses and showed only very modest decreases in DTG susceptibility (137). Importantly, the combination of N155H and R263K has never been documented in individuals receiving DTG therapy but previous work has shown that these two mutations may not be incompatible in the context of the virus in tissue culture (159). The current study suggests that this combination of mutations will not yield high-level resistance against DTG.

4.6 Acknowledgements

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Chapter 5

The effect of polymorphic position 157 on R263K-mediated dolutegravir resistance

This chapter was adapted from the following manuscript published in the Journal of Antimicrobial Chemotherapy in 2016: “The polymorphic substitution E157Q in HIV-1 integrase increases R263K-mediated dolutegravir resistance and decreases DNA binding activity” by Kaitlin Anstett, Robert Fusco, Vincent Cutillas, Thibault Mesplede, and Mark A. Wainberg. K.A. performed 90% of the experiments and data analysis, partially supervised by T.M. and aided by R.F. and V.C. as trainees under her supervision. M.A.W. supervised and advised the project and obtained funding for its pursuit. K.A., T.M., and M.A.W. wrote the manuscript.

5.1 Preface

In Chapter 4 we explored the possibility of combining the N155H and R263K resistance pathways, and found that secondary resistance mutations common to N155H were not compensatory in the background of this double mutant. However, one of the so-called compensatory mutations continued to intrigue us. E157Q is a polymorphism that is present in up to 10% of the viral population, depending on subtype. It has also been associated with resistance to the earlier INSTIs raltegravir and elvitegravir (237).

It was reported that a highly treatment-experienced patient failed RAL, then subsequently DTG, with E157Q as the only resistance-associated change detected following failure under DTG. The authors characterized the patient-derived IN as having high enzymatic activity and as being highly resistant against DTG (187). This study was of particular interest because it was the first to associate E157Q with resistance to DTG, however as the authors did not report the patient-derived sequence that was used in their assays, it remained unclear whether the observed phenotype was only due to the polymorphic change. Other polymorphisms such as M50I or S119R have been shown to

also contribute to drug resistance but are not always detected by phenotyping algorithms given the fact that they are naturally polymorphic. Thus, we decided to investigate the potential of the E157Q substitution as a significant pathway towards DTG resistance both on its own, and in combination with R263K. As a polymorphism it is possible that E157Q could be present at baseline before treatment initiation with DTG, even for INSTI-naïve individuals, making the question of whether this substitution might affect the development of R263K-mediated DTG resistance ever more significant.

5.1.2 Abstract

Objectives: The E157Q substitution in HIV-1 integrase (IN) is a relatively common natural polymorphism associated with HIV resistance against integrase strand transfer inhibitors (INSTIs). Although R263K is the most common resistance substitution for the INSTI dolutegravir, an INSTI treatment-experienced individual recently failed dolutegravir-based therapy with E157Q being the only resistance-associated change reported. Given that different resistance pathways can sometimes synergize to confer high levels of resistance against antiretroviral drugs, we studied the effects of E157Q in association with R263K. Because Glu157 is thought to lie within the binding site of HIV IN DNA binding inhibitors such as FZ41, we also evaluated DNA binding activity and resistance to IN inhibitors in the presence of E157Q.

Methods: Purified recombinant IN proteins were assessed in cell free assays for their strand transfer and DNA binding activities. NL4.3 viral stocks harbouring IN mutations were generated and characterized in the presence and absence of IN inhibitors in tissue culture.

Results: E157Q alone had little if any effect on the biochemical activity of integrase, and partially restored the activity of R263K-containing IN. The E157Q/R263K double viral mutant displayed equivalent infectiousness in culture as wild-type, while increasing resistance to dolutegravir by 10-fold compared to lower-level resistance associated with R263K alone. None of the mutations tested showed significant resistance to either raltegravir or to FZ41.

Conclusions: This study shows that E157Q may act as a compensatory mutation for R263K. Since E157Q is a natural polymorphism present in 1-10% of HIV positive individuals, it may be of particular importance for patients receiving INSTI therapy.

5.2 Introduction

Human immunodeficiency virus 1 (HIV-1) is a highly heterogeneous virus, existing as a quasispecies of different variants both within a single patient and at a population level (238). This is due to the error-prone reverse transcriptase (RT) enzyme of HIV, which frequently inserts incorrect nucleotides during reverse transcription, leading to the generation of multiple mutations, some with the potential to confer resistance against antiretroviral drugs (ARVs) (93).

Although resistance has emerged in patients for every currently available ARV class, the integrase strand transfer inhibitor (INSTI) dolutegravir has yet to be shown to select for resistance mutations in treatment-naïve individuals, unlike the earlier drugs of this class, raltegravir and elvitegravir (110, 130, 229). These latter drugs can initially select for primary resistance mutations that cause drug resistance at the expense of viral replicative capacity and later for compensatory mutations that can restore replication capacity while further increasing the level of drug resistance (115). When used as an

initial INSTI in a highly treatment-experienced patient population, dolutegravir did not select for classical INSTI primary resistance mutations; instead, the R263K substitution has been observed in treatment-experienced individuals who failed therapy with this drug (113). We have also observed this substitution during tissue culture selections studies with HIV-infected cells passaged in increasing concentrations of dolutegravir (135). R263K decreases both viral replicative capacity and enzymatic strand transfer activity by 20-30% while conferring 2-5 fold resistance to dolutegravir (169).

It was recently reported that a patient failed therapy with raltegravir, and subsequently dolutegravir, with only one known resistance-associated substitution present in integrase- E157Q (187). Although considered a polymorphic substitution, E157Q has long been implicated in clinical INSTI resistance (132). We previously characterized E157Q in the context of the N155H/R263K HIV-1 subtype B double mutant and showed that E157Q did not have a restorative effect in this background (160).

The present study was designed to determine what effect, if any, E157Q might have on dolutegravir resistance, viral infectivity, and integrase strand transfer activity. We also assessed the effect of E157Q together with R263K. The results show that E157Q acts as a compensatory mutation, increasing enzymatic activity, infectivity and dolutegravir resistance in a R263K-containing background, even though this substitution has little effect on its own on integrase catalytic activity and infectivity in the absence of dolutegravir while also rendering the virus hypersensitive to this inhibitor.

We also investigated the effects of E157Q on IN DNA binding activity and susceptibility to a DNA binding inhibitor in tissue culture, in part because we recently identified E157Q as a putative interaction domain with the HIV integrase DNA binding

inhibitor FZ41 (215). However, E157Q did not have any significant effect on either DNA binding or resistance to FZ41, alone or in combination with R263K.

These results suggest that the natural polymorphism E157Q may partially compensate for impairment to the integrase protein conferred by the dolutegravir resistance mutation R263K, while increasing levels of drug resistance, and this may have implications for the use of dolutegravir in some HIV-infected individuals.

5.3 Materials and Methods

5.3.1 Experimental design

Our objectives were to evaluate the effects of the E157Q polymorphism on the biochemical activities of purified recombinant integrase in cell free assays as well as on viral replication in the presence or absence of integrase inhibitors in tissue culture. We studied both the effect of E157Q alone and in combination with the R263K dolutegravir mutation, compared to wild-type and R263K alone.

5.3.2 Cells and reagents

TZM-bl and 293T cells were cultured as reported previously in Chapter 2 Section 2.3.2 and (135). Merck & Co., Inc., ViiV Healthcare Ltd., and LBPA, ENS Cachan, CNRS supplied raltegravir, dolutegravir, and FZ41 (CID 5481653), respectively.

5.3.3 Integrase strand-transfer activity assay

Site-directed mutagenesis was performed to create both pET15b$_{E157Q}$ and pET15b$_{E157Q/R263K}$ plasmids as previously described in Chapter 2 Section 2.3.7 for the pET15b$_{R263K}$ integrase subtype B expression vector using the following primers: sense: 5'GTCAAGGAGTAATAGAATCTATGAATAACAGTTAAAGAAAATTATAGGACAGGATA GAG3' and antisense: 5'CTCTTACCTGTCCTATAATTTTCTTTAACTGTTTATTCATAGATTCTATTACTCCTTG

AC3' (169). The expression and purification of recombinant integrase proteins has also been described previously in Chapter 2 Section 2.3.11 and (135). Strand transfer assays were performed as previously published and described in Chapter 3 Section 3.3.3 (169). Briefly, DNA Bind 96-well plates (Corning) were coated with HIV-1 pre-processed long terminal repeat (LTR) DNA at 4°C for 48 h and then incubated with 600nM purified proteins. Increasing concentrations of biotinylated target DNA were then added to the plates and the reaction was allowed to occur for 1 h at 37°C. Europium (Eu)-labelled streptavidin, which stably bound the biotin tag of the target DNA molecules, was added and strand transfer activity was quantified via Eu fluorescence.

5.3.4 Integrase DNA binding activity assay

DNA binding activity assays were performed as described previously using purified recombinant proteins (165, 239). The entire protocol was conducted at4˚C. High Bind black 96-well plates (Corning) were coated with 600nM integrase proteins at for 16 h in 50% PBS. Unbound proteins were then removed by washing twice with PBS, then blocked with 5% BSA in 50% PBS. After blocking, the plates were washed twice with PBS and once with DNA binding buffer (20mM MOPS pH 7.2, 20mM NaCl, 7.5mM $MgCl_2$, 5mM DTT). Fluorescently labelled RhoR-LTR duplexes HIV-1 DNA (sense: 5'-CTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-30', antisense: 5'-/Rhodamine-XN/ACTGCTAGAGATTTTCCACACTGACTAAAAG-3') were serially diluted 6X from 6 uM to 0.1uM in DNA binding buffer, then added to the plates and binding was allowed for 1 hr at room temperature in the dark. Reactions were then washed 3X with PBS to remove unbound DNA, and DNA binding activity was measured via RhoR fluorescence at 544nm.

5.3.5 Generation of NL4.3 HIV-1 viral clones

The generation of the pNL4.3$_{IN(R263K)}$ plasmid has been reported previously and similar methods were used to generate the pNL4.3$_{IN(E157Q)}$ and pNL4.3$_{IN(E157Q/R263K)}$ plasmids using the primers described above (135). Viral stocks were produced as described previously in Chapter 2 Section 2.3.7 and (135). 293T cells were transfected with plasmid DNA using Lipofectamine 2000. At 48 h after transfection, cell culture fluids were collected and filtered at 0.45μm to remove cell debris. Viral stocks were then aliquoted and stored at -80°C. Quantification of infectious viral particles was on the basis of measuring cell-free RT activity.

5.3.6 HIV inhibition assay

HIV susceptibilities to dolutegravir, raltegravir and FZ41 were measured by infection of 30,000 TZM-bl cells with 100 000 RT units of each virus in the presence of serial drug dilutions. After 48 h, cells were lysed and luciferase production was measured using the Luciferase Assay System (Promega, Madison, WI, Canada).

5.3.7 HIV infectivity assay

HIV-1 infectivity was measured through the infection of 30 000 TZM-bl cells per well using serial 1:4 dilutions of each virus. Levels of infection were measured as described above.

5.3.8 Statistical analysis

Each experiment was performed with at least two biological replicates in triplicate (N=6). Biochemical estimates of strand-transfer activity were normalized to wild-type activity at 1600nM protein (Fig. 5.1A) or 128nM target DNA (Fig. 5.1B). DNA binding activity was normalized to wild-type at 6 μM LTR DNA (Fig. 5.2A). Relative EC_{50} was normalized to wild-type EC_{50} (Table 5.1). Fold change (FC) was normalized to wild-type IC_{50} (Table 5.2). K_M, V_{max}, EC_{50}, IC_{50}, standard error of the mean (SEM), and 95% confidence intervals

(95% CI) were calculated using Prism 5.0 Software. All figures were visualized by Prism 5.0 Software. Student's *t*-tests were performed using the OpenEpi toolkit, accessible free online at www.openepi.com.

5.4 Results

5.4.1 E157Q partially restored the strand transfer activity of R263K-containing integrase

Figure 5.1 displays the strand transfer activity of wild-type, E157Q, R263K, and E157Q/R263K-containing HIV-1 integrase subtype B proteins as a function of protein (Fig. 5.1A) or target DNA substrate (Fig. 5.1B) concentrations. Consistent with previous studies all proteins were maximally active at 400 nM (160, 165). It is evident from both panels that the polymorphic E157Q substitution had no effect on the observed enzyme activity since the curves generated are similar to that of the wild-type enzyme. As we have previously reported, the R263K-containing integrase was impaired in strand transfer activity at every concentration of protein or substrate tested; however the double E157Q/R263K mutant displayed an activity that was intermediate between wild-type or E157Q and R263K (159, 168, 169, 228). Figure 5.1C displays the affinity of the integrase enzymes for the DNA substrate as the kinetic constant K_M, where a lower value indicates a higher affinity (240). We have shown previously that IN strand transfer K_M strongly correlates with viral infectivity and replicative capacity and is thus a good indicator of the possible phenotypic effects of various substitutions (135, 168). The presence of R263K resulted in a sharp increase in the observed K_M, which was partially restored in the case of the double mutant. When the measure of enzyme proficiency, V_{max}/K_M, where V_{max} represents the maximal strand transfer activity of the protein, was taken into consideration (Figure 5.1D), the same trend was observed. Thus, the polymorphic substitution E157Q

had little effect on its own but improved the strand transfer activity of integrase enzymes containing R263K.

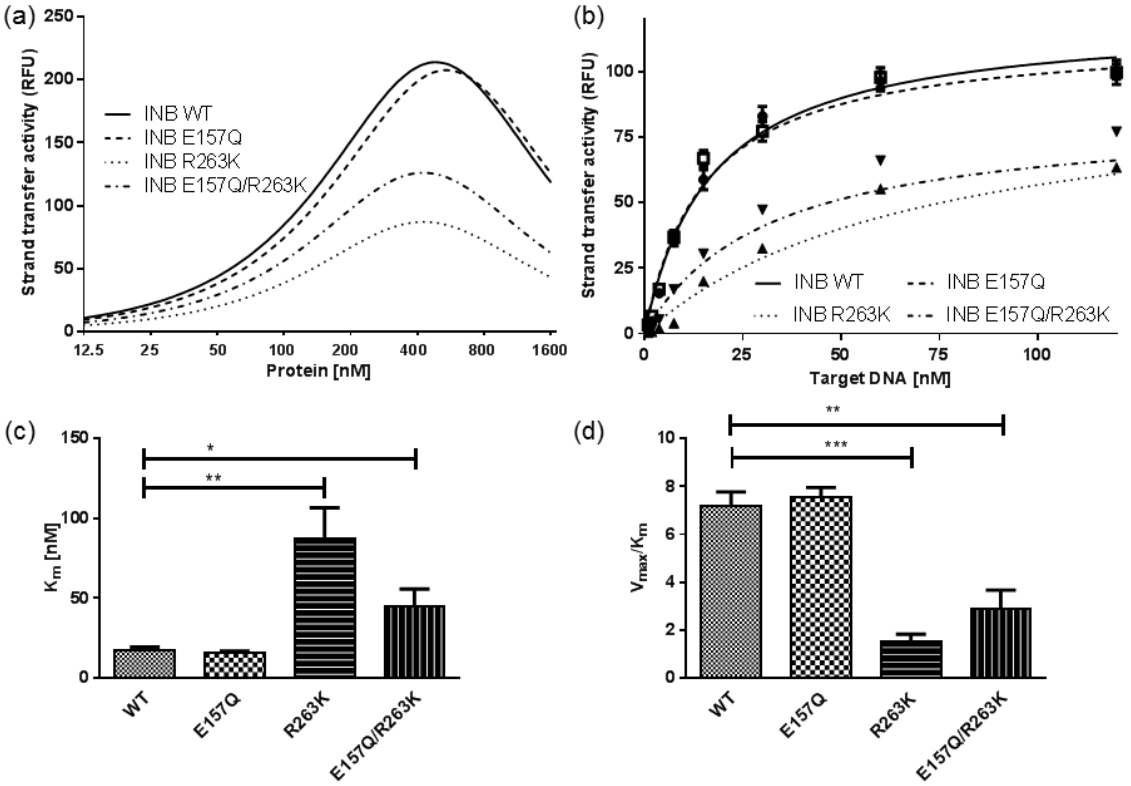

Figure 5.1: Strand-transfer activities of purified recombinant integrase proteins. Biotinylated target DNA incorporation was measured by Eu-streptavidin fluorescence as a function of (a) protein concentration or (b) target DNA concentration. (c) K_M values for each integrase mutation were obtained from the curves in (b). (d) Enzyme proficiency was quantified by dividing V_{max} (taken from plateau in (b)) by K_M. Error bars display standard error of the mean (SEM). * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$ by Student's *t*-test.

5.4.2 DNA binding activity of integrase is restored to wild-type levels upon the addition of E157Q to R263K

We recently identified position Glu157 as a putative interactive residue with the integrase DNA binding inhibitor FZ41; therefore, we evaluated the effect of the E157Q substitution on the *in vitro* DNA binding activity of integrase (215). Figure 5.2A shows the DNA binding activity of each protein studied as a function of fluorescently labelled HIV LTR DNA concentration. All the enzymes studied appeared to have similar activities. However, K_M

calculations showed that R263K alone led to an improved DNA binding activity compared to the other proteins (Fig. 5.2B) and this increase was statistically significant as shown in enzyme efficiency studies (Fig. 5.2C). Thus, the polymorphic substitution E157Q acts to decrease DNA binding levels of the R263K-containing mutant.

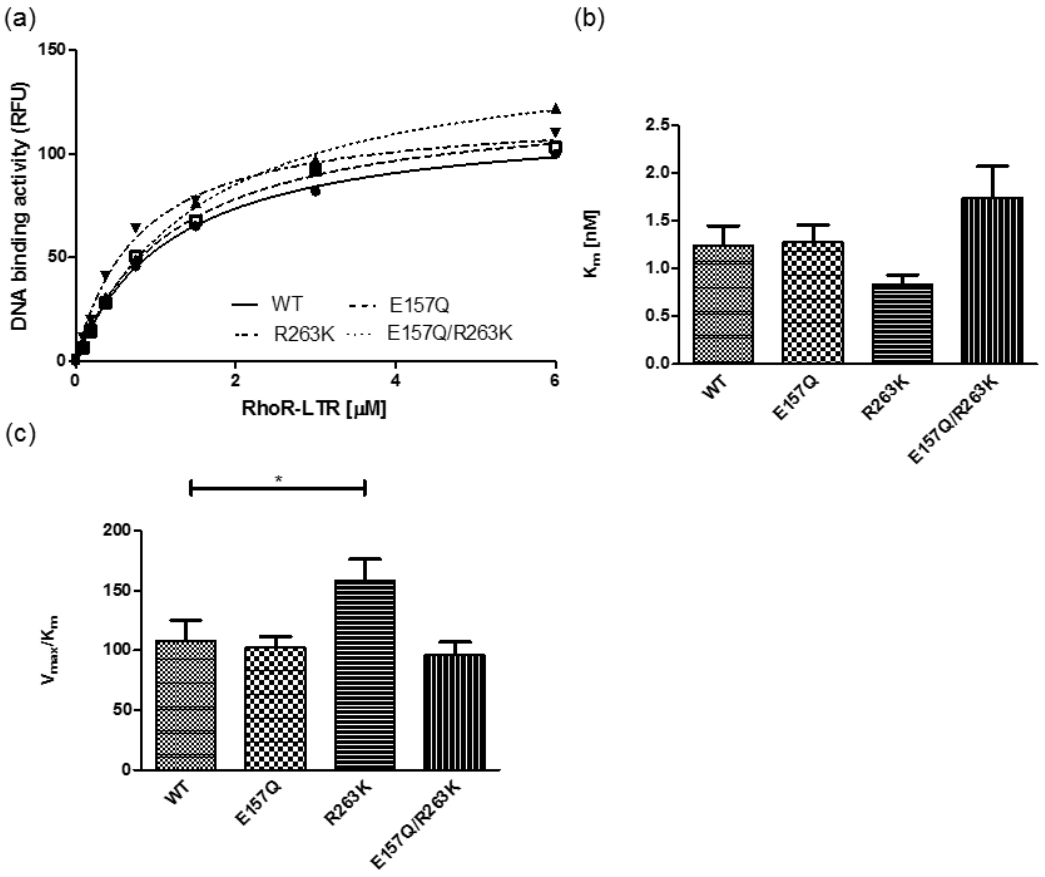

Figure 5.2: Cell-free DNA binding activities of purified recombinant integrase proteins. (a) Enzymatic activity as a function of increasing HIV LTR DNA concentration. (b) K_M values for each mutation were obtained from the curves in (a). (c) Enzyme proficiency was quantified by dividing V_{max} (taken from plateau in (a)) by K_M. Error bars display standard error of the mean (SEM). * = $p \leq 0.05$ by Student's *t*-test.

5.4.3 E157Q-containing NL4.3 viruses are infectious

Table 5.1 displays the infectivity of subtype B NL4.3 viral stocks in TZM-bl reporter cells as a function of increasing RT activity – these results are also displayed in Figure 5.3. Although the E157Q/R263K double mutant displayed a lower EC_{50} value than wild-type,

this trend did not reach statistical significance. As has been shown in the past, the R263K substitution resulted in a ~30% decrease in HIV-1 infectivity relative to wild-type (135, 159, 168, 169, 228). This decrease was completely restored in the case of the E157Q/R263K double mutant, a finding that is consistent with the biochemical results presented here and further showing that E157Q can compensate for the R263K mutation.

Genotype	EC_{50} relative to WT	95% CI
WT	1.00	0.64–1.56
E157Q	1.23	1.02–1.50
R263K	2.67*	1.99–3.58
E157Q/R263K	0.66	0.53–0.82

Table 5.1: Relative EC_{50} values for NL4.3 viral stocks in TZM-bl cells. EC_{50} relative to wild-type. **denotes an EC_{50} value significantly different than wild-type by Student's *t*-test ($p \leq 0.01$).

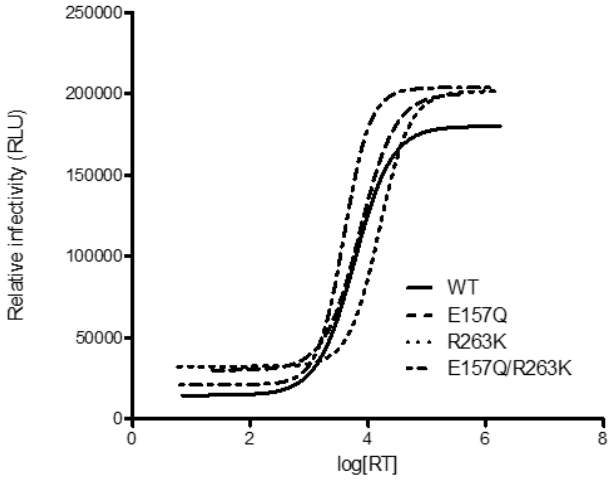

Figure 5.3: E157Q-containing viruses are infectious. Infectivity in TZM-bl cells of WT, E157Q, R263K, and E157Q/R263K NL4.3 viruses measured by luciferase luminescence as a function of cell-free reverse transcriptase (RT) activity.

5.4.4 E157Q is hypersensitive to dolutegravir but enhances R263K-mediated resistance by 10-fold

In Figure 5.4A the infectivity curves of each virus studied in increasing concentrations of DTG are shown and Table 5.2 shows the IC_{50} values for dolutegravir of these viruses.

The E157Q virus was hypersensitive to dolutegravir, displaying an IC_{50} of 0.43 nM, which was 100-fold lower than wild-type (52.1 nM). The R263K virus displayed low levels of dolutegravir resistance, consistent with previous reports (135, 159, 168, 169, 228). However, the combination of E157Q and R263K increased dolutegravir resistance by 20- and 10-fold compared to the wild-type and R263K enzymes respectively, with the IC_{50} for the double mutant being elevated to ~1μM.

	Dolutegravir			Raltegravir			FZ41		
Genotype	IC_{50} (nM)	95% CI	fold change relative to WT	IC_{50} (nM)	95% CI	fold change relative to WT	IC_{50} (nM)	95% CI	fold change relative to WT
WT	52.10	16.83–161.2	1.00	23.59	15.28–36.41	1.00	9029	8804–9259	1.00
E157Q	0.4277	0.304–0.602	0.01*†	10.70	7.356–15.55	0.454*†	7605	6419–9011	0.84*
R263K	106.1	20.73–543.4	2.04	4.131	3.124–5.463	0.175*	8114	7590–8674	0.90*
E157Q/R263K	1035	407.3–2632	19.9*†	38.56	18.14–81.96	1.63†	7068	6147–8126	0.78*

Table 5.2: IC_{50} values for NL4.3 viral stocks in TZM-bl cells for dolutegravir (DTG), raltegravir (RAL), and FZ41.95% confidence intervals (95% CI) and fold-change relative to wild-type (FC) are shown. * Denotes values significantly different than wild-type by Student's *t*-test ($p \leq 0.05$). † Denotes values significantly different than R263K by Student's *t*-test ($p \leq 0.05$).

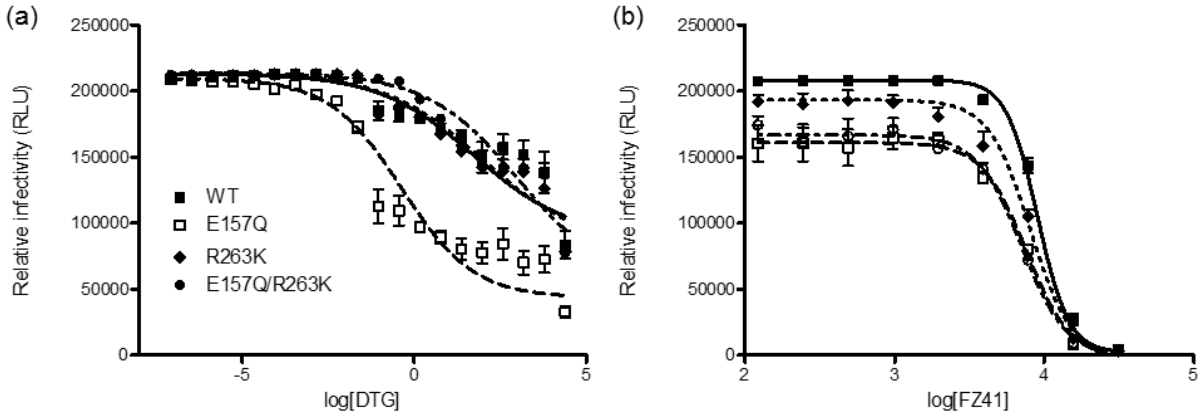

Figure 5.4: Infectivity of WT, E157Q, R263K, and E157Q/R263K NL4.3 in the presence of increasing concentrations of (a) dolutegravir (DTG) and (b) FZ41. Infectivity was measured by luciferase luminescence as a function of increasing drug concentration.

5.4.5 Neither E157Q nor R263K alone or in combination decreased susceptibility to raltegravir or FZ41

Also summarized in Figure 5.4 (B) and Table 5.2 are IC_{50} values derived for each virus for raltegravir. Despite previous reports that identified E157Q as raltegravir resistance-associated substitution in patients failing treatment with this drug, we did not observe a significant increase in IC_{50} compared to wild-type (187, 235). The presence of E157Q also had little effect when added to a R263K-containing background; however, it did significantly increase levels of raltegravir resistance compared to R263K alone.

Table 5.2 also displays the infectivity of viruses in response to the integrase-DNA binding inhibitor FZ41. Although we had previously identified position 157 in integrase as a potential interactor with this inhibitor, the E157Q mutation, alone or in combination with R263K, did not display any increase in IC_{50} compared to the wild-type virus (215).

5.5 Discussion

In this study, we addressed the potential role of the E157Q polymorphic HIV-1 substitution in individuals treated with dolutegravir. A previous report had suggested that a patient failed raltegravir, and subsequently dolutegravir, with only E157Q in integrase being present. There was strong indication that this patient was adherent to his ART regimen due to the high plasma concentration of medication and the lack of resistance to the background regimen (187). The authors concluded that HIV bearing this substitution was more fit than wild-type and also highly resistant to both of the above-mentioned INSTIs. In contrast, our results show that E157Q on its own has only minimal impact on integrase, both in terms of enzyme activity and viral infectivity. We have also shown that E157Q on its own confers hypersensitivity to dolutegravir without significantly altering susceptibility to raltegravir. Discrepancies between our and previous studies can be explained in part by differences in experimental protocols, since we uniquely evaluated the contribution of

each mutation by site-directed mutagenesis using the NL4.3 backbone while the other studies utilized the entire patient-derived IN sequence. Considerable viral sequence evolution can occur during treatment failure and this may have contributed to the observed phenotype in the previous report (188).

Here, we show that the E157Q polymorphism has no significant effect on either integrase strand transfer activity or DNA binding affinity of HIV-1 subtype B integrase protein. However, as shown here, E157Q is able to partially restore deficits in integrase enzymatic activity caused by the R263K substitution, thereby acting as a secondary, compensatory mutation. Again, this is consistent with the fact that E157Q can function as a compensatory mutation for primary raltegravir/elvitegravir resistance mutation N155H (160, 177). Some reports have stated that E157Q is deleterious for viral replication without conferring much drug resistance, while others claim that the reverse is true (187, 234, 235). We recently reported that E157Q had beneficial effects on some enzymatic activities of the integrase protein, while also conferring moderate dolutegravir resistance when combined with N155H and R263K in biochemical assays (160). It is also worth noting that the Stanford Drug Resistance Database does not consider E157Q to be a major INSTI resistance determinant (115).

There has yet to be a report of E157Q in combination with R263K in the clinic, despite high levels of dolutegravir resistance and viral replication capacity as shown in the current study. Interestingly the E157Q substitution in isolation rendered the virus hypersensitive to dolutegravir. This may be because this residue is located near the active site of the protein, and while we have shown that it does not have a significant effect on enzymatic activity, E157Q may affect the binding of dolutegravir to this region. As we

have shown E157Q to be hypersensitive to dolutegravir, it follows that this substitution would not arise first in response to drug pressure, but this does not explain its absence as a secondary mutation after R263K. Furthermore, more than five years of dolutegravir selection in our laboratory with the R263K-containing virus has not yet led to any compensatory mutation for R263K (ongoing). This supports the hypothesis that R263K may represent an evolutionary dead-end pathway for the virus, from which it may be unable to escape. This may be due to deleterious effects of R263K on HIV RT activity, which may, in turn, influence the mutational capability of R263K-containing virus; investigations are currently underway to address this question. The fate of HIV-1 bearing E157Q in dolutegravir selections is also currently under evaluation. Of course, first-line dolutegravir resistance has so far been a rare occurrence and hence further monitoring of patients on dolutegravir therapy is necessary to indicate whether E157Q will ultimately be shown to have clinical relevance or not (241).

E157Q also fully compensated for the infectious deficit of R263K when the two substitutions were combined in NL4.3, while having no significant effect on its own. The E157Q/R263K double mutant also displayed enhanced dolutegravir resistance with a calculated IC_{50} of ~1 μM compared to R263K alone with an IC_{50} of 106.1 nM, which represents a 20-fold decrease in susceptibility compared to wild-type. This finding may have implications for the treatment of HIV-positive individuals with dolutegravir, given that E157Q is naturally present in 1-10% of untreated individuals, depending on subtype (237). Thus, the presence of E157Q at baseline could potentially have a negative connotation. This is dependent on whether the hypersensitivity of this mutant to dolutegravir would be recapitulated *in vivo.*

E157Q was also recently identified as a putative interactor with a new class of HIV integrase inhibitors that specifically target the DNA binding activity of the enzyme (215). Therefore, we also wanted to evaluate whether this polymorphic substitution could have effects on resistance to this class of inhibitors *in vitro*, as this would have implications for the further clinical development of compounds of this class. However, we found that E157Q had little effect on the DNA binding activity of purified recombinant integrase, and this translated to a lack of resistance to FZ41 in tissue culture experiments.

In conclusion, this study highlights the importance of genetic background in viral evolution under drug pressure. We have shown the compatibility of the dolutegravir R263K mutation with the polymorphic substitution E157Q. This combination appeared replication competent and yielded higher level resistance to dolutegravir than either mutation on its own. The combination of the E157Q and R263K mutations should be monitored in clinical practice to determine whether they could provide a mechanism through which HIV might be able to escape dolutegravir pressure in the clinic.

5.6 Acknowledgements

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Chapter 6
Discussion

The overarching aim of this thesis was to gain a better understanding of the R263K dolutegravir resistance pathway. We wished to uncover the mechanisms whereby this substitution causes resistance to DTG, whether this process is compatible with the mechanisms of first-generation INSTI resistance pathways, and whether it might be possible to engineer a highly resistant HIV-1 variant via the R263K pathway. These questions are important; although antiretroviral therapy has revolutionized the treatment of HIV-positive individuals, drug resistance has been documented in patients for every inhibitor currently on the market (88, 210). This complicates the long-term treatment of HIV infection and puts the survival of many patients in jeopardy over time. The pathways that provide resistance to first-generation INSTIs are straight-forward: these alterations cause perturbations to the enzyme active site, thereby affecting the binding of these competitive inhibitors (183). However, position 263 is located in the C-terminus of the protein, not the catalytic core domain, and thus its influence on inhibitor binding and enzymatic activity is not as obvious. DTG is one of the best antiretrovirals available to date as very few patients have failed treatment and DTG has a high genetic barrier to resistance (134). However, it is not perfect. Understanding the mechanism by which the R263K substitution imparts DTG resistance is a first step toward overcoming said resistance, to designing better compounds in the future, and to intelligently creating combination therapies better able to restrain HIV-1 with this mutation.

6.1 The role of acetylation in HIV-1 INSTI resistance

The effect of post-translational modifications of proteins has long been known to influence various cellular processes, such as genomic transcription and stress responses (242). It

has also been established that the post-translational modifications of various HIV-1 proteins are essential for proper, productive infection (243). HIV integrase in particular has been shown to be ubiquitinated, SUMOylated, methylated, and acetylated (63). The latter appears to be particularly important for IN DNA and RNA binding, and for enzymatic activity in general (61, 67). Our lab identified the R263K resistance substitution proximal to the three lysine residues whose acetylation helps mediates nucleic acid interactions and hypothesized that this amino acid change might exert an effect on the acetylation of the CTD of IN. Because this substitution introduces another lysine into the region, it is possible that position 263 is also post-translationally modified, either in addition to or at the expense of positions 264, 266, and/or 273. This residue is also within the binding interface for both p300 and KAP1 and therefore R263K could have affected the affinity of these cellular proteins for IN (63). Although we could not substantiate or refute the former, we were able to show in Chapter 2 that R263K-containing IN does interact with higher affinity with KAP1 than does WT protein. Since both proteins showed the same levels of affinity for p300, this suggests that IN that contains R263K is more readily deacetylated, which should correlate to lowered nucleic acid binding in cells. Why this would be advantageous to the virus is not clear. As INSTIs bind in the active site of the enzyme and interact with both the protein and viral DNA, the ability to dissociate more readily from DNA may translate to an increased ability to dissociate from the inhibitor as well. Conversely, residues 264 and 266 have also been linked to IN multimerization (62). We cannot rule out an effect of deacetylation of these residues on the formation and/or stability of the enzymatically active IN tetramer.

Chapter 2 also explored the role of cellular HATs and HDACs over the course of HIV-1 infection, both in the presence and absence of INSTIs. We found that inhibition of cellular acetylation decreased the IC_{50} of WT virus for DTG as well as for a RAL resistant variant while having no effect on the R263K-containing virus. This effect was limited to second-generation INSTIs, as no change in IC_{50} values was seen for either RAL or 3TC. As discussed above, the IN_{R263K} is likely more readily decetylated than the WT enzyme; thus, a diminution of cellular acetylation might have less of an effect on this mutant. The question remains, however, why inhibiting acetylation sensitizes HIV-1 to DTG, CTG, and BIC. It may be that lowering levels of acetylation also decreases the activity of IN, resulting in more efficient inhibition by second-generation INSTIs. It would then follow that RAL, which displays a shorter binding half-life than the newer drugs of this class, may be less affected by the modulation of cellular acetylation (163). The R263K-containing virus was sensitized to inhibition by BIC upon HAT inhibition, which points to differences between this inhibitor and other second-generation INSTIs. Clearly, further experimentation is needed to confirm the mechanism of INSTI sensitization upon HAT inhibition.

We also observed an effect of both HDAC and HAT mediated inhibition in virus producer cells. When WT HIV was produced in the presence of an HDAC inhibitor, it displayed a decreased peak of infection, while HAT inhibition had no observable effect on virus production. The opposite was true for HIV_{R263K}; which was unaffected by HDAC inhibition; indeed, HAT inhibitors greatly enhanced the peak replication of this virus. These results suggest that the regulation of acetylation is important during HIV particle production. Because the R263K-variant has a tighter association with the HDAC I complex than WT virus, it could be less affected by HDAC inhibition. Conversely, HAT

inhibition greatly improved the replication of HIV_{R263K} while HIV_{WT} remained unaffected, which suggests that the fitness defect of the R263K mutant may be tied to HAT activity. The modulation of cellular acetylation also affected HIV-1 production both in target and producer cells, signalling that the requirements for IN acetylation and deacetylation may vary over the course of infection, and that IN is likely post-translationally modified both before and after infection of a target cell. The HDAC I complex has been shown to be recruited into the HIV-1 virion *in vitro.* Furthermore, the proper function of this complex in producer cells was important for HIV infectivity in target cells, consistent with our results (244). There are however no reports of the recruitment of p300 into the HIV-1 virion.

The experiments in Chapter 2 have raised several interesting avenues of research regarding the acetylation of HIV IN and its effects on the retroviral life cycle. Although we did establish that the regulation of cellular acetylation has major effects on HIV-1 biology and resistance to INSTIs, and that this effect is modulated at least in part by interactions with the cellular protein KAP1, we still do not have a clear understanding of how the R263K substitution confers resistance to DTG and other second-generation INSTIs; this continues to be an area of active research in our laboratory.

6.2 The (in)compatibility of R263K with first-generation INSTI resistance

Due to its activity against many RAL/EVG resistant variants, DTG is a promising salvage therapy for HIV-positive individuals failing treatment with these earlier INSTIs. The VIKING clinical trial showed that the majority of highly treatment-experienced patients could still achieve undetectable viral loads with DTG, even with pre-existing INSTI resistance mutations (188). The individuals that did fail treatment in this study did so

without the development of R263K, as happened with INSTI-naïve patients in the SAILING clinical trial (113). Instead, an accumulation of multiple primary INSTI resistance mutations seemed to be responsible for treatment failure. This led us to question whether or not the R263K resistance pathway was compatible with other primary INSTI resistance pathways.

In Chapter 3, we presented the results of the combination of R263K with primary RAL/EVG resistance mutations, both in enzymatic assays and with viruses in tissue culture. When two substitutions each have a negative effect on viral replication, it follows that the combination of these mutations on a single virus should have a further deleterious effect. This was indeed the case for all the combinations of mutations studied with regard to IN in cell-free assays. Additionally, only one combination, Q148R/R263K, showed any increase in DTG resistance at the protein level compared to R263K alone; however, the virus bearing this combination was severely impacted and replicated quite poorly, making it unlikely to be a concern *in vivo*. The Y143R/R263K variant likewise showed very diminished infectivity. While the combination of E92Q with R263K was relatively infectious, it did not display any increase in DTG IC_{50} compared to R263K alone, and was thus also unlikely to be of concern.

The N155H/R263K variant, however, broke away from this paradigm. Not only was this combination of mutations more fit than either single mutant, it also displayed an increased DTG IC_{50}. Why these two substitutions are complementary is unclear. We established in Chapter 2 that the R263K resistance pathway is distinct from first-generation INSTI resistance pathways in that it acts, at least in part, through an altered interaction with a cellular binding partner of IN. Perhaps, because these two substitutions

act via independent mechanisms, their negative effects on infectivity are not additive. The R263K mutation had been identified prior to our initial DTG selections as a rare secondary EVG resistance mutation, both in tissue culture and in treated patients (171, 177). It is then possible that R263K can also act as a compensatory mutation in this background, but whether through the same or a different mechanism than the one uncovered in Chapter 2, remains unknown.

Around the time published these data, there were few reports associating the N155 pathway with DTG resistance in the clinic (159, 166). Since then, one third of all confirmed failures on DTG in INSTI-naïve patients (2/6) have been associated with the N155H mutation (137). Although we had identified a clinically significant route to DTG resistance, we wanted to move further with this combination of pathways to determine whether or not the addition of N155H to R263K could lead to a hyper resistant variant, as the decrease in susceptibility of the double mutant to DTG compared to WT was only about 3-fold. We had hypothesized previously that the R263K resistance pathway represented an evolutionary dead-end for HIV-1, as this mutation only confers low levels of resistance to DTG while decreasing IN enzymatic activity and viral replicative capacity by ~30% (135, 245). Furthermore, no substitution has been identified that can compensate for the fitness deficit or the increased levels of DTG resistance conferred by R263K (167-169). Thus, the use of DTG and the subsequent selection of R263K may actually be beneficial to patients if it renders the virus less fit and less infectious.

Chapter 4 summarizes our attempts to create a hyper DTG resistant HIV-1 virus by adding N155H-specific secondary mutations to the N155H/R263K background. The rationale was that R263K might compensate for N155H under RAL or EVG pressure, and

that this might happen in the presence of DTG as well. Although some of the substitutions had positive effects on the enzymatic activity of IN in this background, no mutation improved the infectivity or further increased the DTG IC_{50} compared to N155H/R263K alone. In contrast, decreases in susceptibility to DTG occur as secondary resistance mutations are added to the Q148 pathway (162). This may suggest that the accumulation of secondary resistance mutations as a route to DTG resistance is specific only to Q148X mutants. Therefore, prior exposure to first-generation INSTIs (as DTG does not select for Q148X) may be the only way that high levels of DTG resistance can develop in HIV-positive individuals.

In Chapter 5 we further explored the possibility of creating a highly DTG-resistant HIV-1 variant through the R263K pathway. We combined the polymorphic substitution E157Q with R263K and found that the former could compensate for the fitness defect of the latter, while also increasing DTG IC_{50} by 10-fold. This Chapter was inspired by the report of a highly treatment-experienced patient failing therapy with RAL, then subsequently with DTG, with E157Q as the only reported resistance-associated change at the time of DTG failure (187). In this study, the authors characterized the patient-derived IN as being highly active and possessing high resistance against DTG. Although we corroborated the former finding, we found the E157Q-bearing virus to be hypersensitive to DTG. The difference in our results highlights the advantages and disadvantages of using patient viruses versus laboratory strains. While the former approach was able to accurately characterize the virus that was replicating in the patient, our study was able to discern the specific contribution of the E157Q substitution through comparison with the parental, wild-type laboratory strain.

Chapter 5 raises some important questions regarding DTG resistance; is the E157Q pathway a significant DTG resistance pathway, and can E157Q and R263K be selected together? In the NL4.3 background, we have shown that the E157Q mutant is hypersensitive to DTG. This may be because the substitution is located within the CCD of IN and may therefore affect the binding of this competitive inhibitor – this is likely the mechanism through which the change provides resistance to RAL and EVG, although there are many conflicting reports on whether E157Q is actually a beneficial substitution for the virus, and what levels of resistance, if any, it confers (234, 235). It may be that E157Q is an ambiguous change, whose effects vary drastically depending on the background HIV-1 genotype. This would explain its wide range in prevalence as a polymorphism (1-10%) depending on subtype (237). Due to the observed hypersensitivity, it is unlikely that E157Q would be selected under DTG pressure, and, as has been stated previously, no compensatory mutation has yet to be selected after the development of R263K in our ongoing tissue culture selections. Thus, the only real concern regarding this position is if it is present at treatment initiation with DTG, either through previous INSTI exposure or as a baseline polymorphism, because it would be highly evolutionarily advantageous for the virus to select for R263K under these conditions. Investigations are currently under way in our laboratory to address this possibility. We are performing DTG selections studies in CMBCs (as detailed in Chapter 3) with $NL4.3_{IN(E157Q)}$ and patient-derived samples, all containing the substitution, to determine whether high levels of resistance may evolve under drug pressure. The results of these experiments will have significant clinical impact as they will point to the likelihood of DTG failures via this INSTI resistance pathway.

6.3 The future of dolutegravir resistance

DTG has been approved for use for less than four years and there are not many data on patient treatment failures (181). The patterns that are emerging, however, appear to be different from those of RAL and EVG. Whereas the latter two inhibitors select for essentially the same resistance substitutions in tissue culture as in the clinic, the selection of DTG resistance appears to vary widely based on the patient population and the viruses present (139). Only 6 INSTI-naïve patients have been reported thus far as having failed DTG-based therapies, but whether this is further evidence of the efficacy of such treatments, a reporting bias, or a reflection of increased use of DTG as a salvage therapy for RAL/EVG as opposed to in first-line usage is unclear.

There have been many recent reports of seemingly sporadic selection of DTG resistance in INSTI-experienced patients (136, 166, 187). There have been reports as well, as in the VIKING clinical trial, of patients that failed DTG because of an accumulation of first-generation INSTI resistance mutations; however, other individuals have experienced virological failure with only single amino acid changes reported, none of them being R263K. This is worrisome because it suggests that DTG resistance in some experienced patient populations may occur through the sporadic selection of seemingly random changes which cannot be predicted. Alternatively, these instances could reflect a caveat in the way that resistance data is reported. If only the changes that are already known to be associated with resistance are reported, there is a risk of overlooking a novel resistance-associated change that could be clinically significant. For example, DTG failure was reported in a RAL-experienced patient with E157Q as the only resistance-associated change (187). High fold- resistance for the patient-derived IN enzyme

compared to WT was also reported; however, we subsequently showed that a laboratory strain bearing the same mutation was hyper-sensitive to DTG (161). It is possible, then, that another change that was not reported in the original study had contributed to the level of DTG susceptibility observed. This highlights potential issues on how HIV-1 resistance data are reported and how this can complicate predictions of drug resistance in HIV-positive individuals.

6.4 Conclusions

Understanding the mechanisms that lead to viral drug resistance is important in the fight against the global HIV pandemic, providing information on how antiretroviral drugs interact with their targets and what alterations to its biology the virus can tolerate in order to evade drug pressure. The INSTIs represent the most recently approved class of ARVs and include DTG, perhaps the most successful ARV to date. DTG possesses a high genetic barrier to resistance and does not select for cross-resistance mutations with the earlier drugs of this class, RAL and EVG (135). By studying the DTG-signature mutation R263K, we hoped to gain insight into the mechanism of action of this novel change and also into the activity of DTG itself. We observed an indirect regulation of DTG susceptibility that the R263K-containing virus appeared to be protected from. This has opened an entirely new avenue of research into how resistance mutations may affect the post-translational regulation of the viral IN protein, and how this in turn affects the biology of this protein, thereby allowing it to evade inhibition by INSTIs. This suggests that it may be possible to use drugs that modify the epigenome of the cell in combination with ARVs in the treatment of drug resistant HIV. These results also have implications for the use of

HDAC inhibitors in so-called “Shock and Kill” strategies to reactivate the latent reservoir, as they suggest that different genotypes of HIV may react very differently to HDAC inhibition. Further investigation is needed in this area but the therapeutic implications of these findings could be important.

We also showed that the DTG-signature mutation R263K is incompatible with most first-generation INSTI resistance mutations. We evaluated scenarios whereby the virus could harbour both pathways simultaneously and showed that this was not likely to be a viable route toward high levels of DTG resistance *in vivo*; this emphasizes the unique character of the R263K resistance pathway and of DTG itself. As DTG has been approved for less than four years, only time will tell whether clinically relevant DTG resistance will occur in first-line therapy or which mutations will be involved. However, our results suggest that such resistance will be very rare and distinct from that seen with other INSTIs. It is our hope that this will translate into better treatment outcomes for HIV-positive individuals worldwide and to a lessening of the global HIV/AIDS disease burden.

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