SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Integration Events in the Life Cycle of HIV-2, and its susceptibility to Lenacapavir and Integrase Strand Transfer Inhibitors

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1. INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is a progressive immunodeficiency disorder caused by human Immunodeficiency viruses type 1 and 2 (HIV-1, HIV-2), leading to $CD4^+$ T cell depletion and increased vulnerability to opportunistic infections. While HIV-1 accounts for the majority of global infections (approximately 39 million cases by 2024), HIV-2 remains less prevalent, primarily in West Africa and select regions of Europe, Asia, and the Americas. HIV-2 is genetically related to HIV-1 with approximately 60% nucleotide identity, but is clinically less virulent, characterized by slower disease progression, reduced transmissibility, and a prolonged asymptomatic phase, possibly linked to unique long terminal repeats (LTRs) regulation and integration site preference. In addition, HIV-2 possesses an accessory protein, viral protein x (Vpx), which enhances infection of myeloid-lineage cells by counteracting host defenses.

A critical step in retroviral replication is the integration of viral DNA into the host genome, catalyzed by the viral integrase and often facilitated by the host factors such as the lens epithelium derived host factor p75 (LEDGF/p75), which promotes integration of HIV-1 into actively transcribed chromatin. While HIV-1 preferentially integrates into active genes, in vivo data suggests that HIV-2 may more frequently target the heterochromatin, potentially contributing to its lower replication rate. The extent of HIV-2 dependence on LEDGF/p75 remains underexplored, and integrase-host interactions represent promising therapeutic targets.

HIV-2 exhibits intrinsic resistance to several antiretroviral classes, including non-nucleoside reverse transcriptase inhibitors (NNRTIs), the fusion inhibitor enfuvirtide, the attachment inhibitor fostemsavir, and many protease inhibitors, but retains susceptibility to nucleotide reverse transcriptase inhibitors (NRTIs), integrase strand transfer inhibitors (INSTIs), and the capsid inhibitor lenacapavir. INSTIs (raltegravir, elvitegravir, dolutegravir, bictegravir, and cabotegravir) block the catalytic activity of integrase enzyme, with second-generation agents (dolutegravir, bictegravir and cabotegravir) showing improved potency and resistance profiles. Lenacapavir, a long-acting capsid inhibitor approved in 2022, has demonstrated strong anti-HIV-1 activity, though data on HIV-2 remain limited.

Therefore, our primary aim was to evaluate the susceptibility of HIV-2 to lenacapavir and INSTIs through cell culture and in vitro based assays. Additionally, we utilized computational

molecular docking to model interaction of HIV-2 integrase with the inhibitors and the capsid with lenacapavir. Our experiments showed that lenacapavir and INSTIs showed excellent efficacy against ROD-based HIV-2, similar to HIV-1.

Due to the limited number of studies on HIV-2 integration mechanisms and its latency period, with existing research yielding inconclusive results, our research sought to fill this gap by investigating the interaction between HIV-2 integrase and LEDGF/p75, and characterize the preferred integration sites of HIV-2 within the host genome. Additionally, we explored additional functional roles of Vpx in HIV-2's lifecycle. Our findings advanced the understanding of HIV-2 integration mechanisms, confirmed interaction of HIV-2 integrase with LEDGF/p75, and showed that Vpx protein may be possibly linked to the down regulation of HIV-1 in dual HIV-1 and 2 infections through the down regulation of a splicing factor important for the functioning of HIV-1 Tat protein.

1.1 HIV-2 epidemiology and genomic organization

HIV-2 is known for its lengthy latency period of 15 – 20 years and a slower rate of CD4 decline and development of AIDS, with most patients remaining asymptomatic for long duration. However, if left untreated, it progresses to AIDS similarly to HIV-1. Majority of the infections are found in West Africa, with over 90% of the cases reported in Sierra Leone, Ivory Coast, Guinea-Bissau, Gambia, Cape Verde and Senegal. The spread of HIV-2 to other regions is attributed to the concept of the West African corridor which saw migrations to America, Middle East, Caribbean, Europe, Asia and diverse African regions like Mozambique and Angola. In Europe, notable case numbers have been observed in France, Portugal and Spain. Where both viruses circulate, patients can be dually infected by both HIV-1 and HIV-2 viruses although there is limited information on the prevalence and mechanism of dual infection.

Structurally, the HIV-2 genome shares the same basic organization as HIV-1, comprising the structural genes gag, pol, and env, as well as regulatory genes tat and rev, and accessory genes vif, vpr, nef, and vpx. These genes are flanked by long terminal repeats (LTRs) on both ends which play crucial roles in transcription and integration. The structural proteins constitute viral architecture, replication enzymes, and envelope glycoproteins. Gag encompasses the matrix,

capsid and nucleocapsid genes important for the integrity of viral structure. Pol encodes the enzymes integrase, reverse transcriptase and protease, all critical for replication. The env gene produces glycoproteins involved in viral host cell entry. On the other hand, regulatory proteins Tat and Rev support transcription of viral genome into RNA transcripts and their export into the cytoplasm. Accessory proteins enhance viral infectivity in the early and late phases of the viral life-cycle. While sharing about 60% sequence identity with HIV-1, HIV-2 differs in its accessory gene profile, notably by encoding viral protein X (Vpx) in place of HIV-1's viral protein U (Vpu).

1.2 Viral protein x

Vpx is a 14–16 kDa unique accessory protein exclusive to HIV-2 and select lineages of simian immunodeficiency viruses (SIVs). It plays a crucial role in antagonizing host restriction factors, particularly Sterile Alpha Motif and Histidine-aspartic domain (HD) containing protein 1 (SAMHD1), which depletes intracellular deoxynucleoside triphosphates (dNTPs), required for reverse transcription. Vpx mediates the proteasomal degradation of SAMHD1 by recruiting it to the DCAF1–CUL4A E3 ubiquitin ligase complex, facilitating efficient reverse transcription in non-dividing cells such as macrophages and dendritic cells. Beyond this, recent evidence suggests that Vpx contributes to the nuclear import of the pre-integration complex and is implicated in modifying host immune responses, making it critical to viral replication and immune evasion. It has also been studied for potential applications in gene therapy due to its ability to enhance lentiviral vector transduction.

1.3 HIV entry and integration

The HIV-2 replication cycle mirrors that of HIV-1, involving a series of well-defined steps: viral entry, reverse transcription, nuclear import, integration, transcription, assembly, and budding. Entry into host cells is mediated by binding of the viral envelope glycoproteins to the CD4 receptor and chemokine co-receptors such as CCR5 and CXCR4, with additional co-receptors including GPR15 and CXCR6 contributing to viral tropism. An important difference between the two virions, is that HIV-2 is known to utilize a wide range of chemokine co-receptors. Once internalized, the viral RNA genome is reverse transcribed into double-stranded DNA, and

together with other host and viral proteins, a large complex called the pre-integration complex (PIC) is formed. The viral integrase enzyme, encoded by the pol gene, orchestrates the integration of viral DNA into the host genome. HIV-2 integrase, akin to its HIV-1's counterpart, comprises of three structural domains; an N-terminal zinc-binding domain, a catalytic core containing the DDE motif, and a C-terminal DNA-binding domain, although polymorphisms in the amino acid residues in HIV-2 integrase may affect enzymatic activity and susceptibility to inhibitors.

During the process, integrase performs two main catalytic reactions: the 3'-processing which removes two nucleotides from each 3' end of the viral DNA, and the strand transfer, which joins the processed viral DNA with the host genome. This reactions require divalent metal ions ((Mg^{2+} or Mn^{2+}) and cellular co-factors such as Lens Epithelium Derived Growth Factor p75 **(**LEDGF/p75).

LEDGF/p75 is a chromatin-associated protein that tethers HIV integrase to active transcription units via its PWWP domain and integrase-binding domain (IBD). This interaction facilitates site-specific integration into gene-rich euchromatic regions, optimizing proviral transcription. In HIV-1, LEDGF/p75 is essential for efficient integration and viral replication. Studies have shown that HIV-1 preferentially integrates into active transcription units, driven by LEDGF/p75, maintaining robust replication and a lowered tendency for latency (Schroder et al., 2002). Only a few studies have reported integration sites of HIV-2 with varying results. An in vitro study analyzing 202 HIV-2 insertion sites found that 82% were located within RefSeq genes, suggesting a preference for gene-dense and GC-rich regions, similar to HIV-1 (MacNeil et al., 2006). However, in vivo studies suggest that HIV-2 integrates more frequently into heterochromatin, which may contribute to its slower replication and lower pathogenicity. In addition, HIV-2's dependency on LEDGF/p75 in integration targeting remains largely unexplored.

1.4 Antiretroviral therapy

The therapeutic management of HIV-2 is hindered by its innate resistance to several antiretroviral drugs designed for HIV-1. It is intrinsically resistant to all non-nucleoside reverse transcriptase inhibitors (NNRTIs), the fusion inhibitor enfuvirtide, and certain protease

inhibitors. However, it remains susceptible to a subset of nucleoside reverse transcriptase inhibitors (NRTIs), some protease inhibitors, and integrase strand transfer inhibitors (INSTIs). INSTIs are among the key drugs used in the management of HIV infection and AIDS. They block the strand transfer step of integration by chelating the active site metal ions of integrase, thereby preventing incorporation of viral DNA into the host genome. While first-generation INSTIs such as raltegravir (RAL), elvitegravir (EVG), exhibit activity against HIV-1, second-generation drugs including dolutegravir (DTG), and bictegravir (BIC) and cabotegravir (CAB) have demonstrated superior efficacy, higher resistance barriers and favorable safety profiles. The efficacy of INSTIs against HIV-2 remains poorly characterized.

HIV-2 can develop resistance to INSTIs through primary mutations (e.g., Q148H/K/R, N155H, Y143C/R) and secondary mutations (e.g., G140S, T97A) within the integrase gene. These mutations alter drug binding at the catalytic site, reducing efficacy of the inhibitor. Second-generation INSTIs generally maintain activity against single or dual mutations, but accumulated mutations can result in cross-resistance. HIV-2 isolates may harbor natural polymorphisms in the integrase that mimic or enhance resistance profiles seen in HIV-1.

Molecular docking studies have provided critical insights into the binding interactions of INSTIs with HIV-2 integrase. These computational methods simulate ligand–protein interactions, allowing for the assessment of binding affinities and the identification of key residues involved in drug resistance. Such analyses have shown that second-generation INSTIs possess greater structural flexibility and binding strength, enabling them to retain inhibitory activity even in the presence of resistance mutations.

Recently, the capsid inhibitor lenacapavir has emerged as a promising long-acting agent with potent activity against both HIV-1 and HIV-2. Lenacapavir targets the highly conserved capsid protein, disrupting multiple stages of the viral life cycle, including capsid disassembly, nuclear import, and virion assembly. Its favorable pharmacokinetic properties allow for subcutaneous administration every six months, offering a significant advantage to adherence. Although clinical data on lenacapavir in HIV-2 infection are limited, in vitro studies suggest broad-spectrum efficacy, including against multidrug-resistant strains. Its novel mechanism and high potency

make it a strong candidate for inclusion in future HIV-2 treatment regimens, particularly in resource-limited settings.

The COVID-19 pandemic further highlighted the potential of drug repurposing, with several antiretrovirals, including INSTIs, evaluated for activity against SARS-CoV-2. Computational studies proposed interactions between INSTIs and viral enzymes such as the RNA-dependent RNA polymerase and helicase, but the results have not translated into a clinical benefit.

2. AIMS

Over the years, research has primarily focused on HIV-1, leaving significant gaps in our understanding of HIV-2. Currently, all approved antiretroviral drugs and inhibitor regimens are developed specifically for HIV-1, with limited understanding of their effectiveness against HIV-2. The susceptibility of HIV-2 to these inhibitors remains poorly characterized, and there are no standardized treatment guidelines tailored to its management. Most existing research on HIV-2 drug efficacy has been limited to in vitro assays or clinical case studies involving patients who have failed combination antiretroviral therapy (cART), leaving significant gaps in evidence-based treatment strategies. Furthermore, the mechanisms underlying HIV-2's integration into the host genome and its association with prolonged latency remain insufficiently explored, with previous studies producing inconclusive findings. In our previous study, we found that Vpx significantly reduced HIV-1's infectivity in a dual HIV-1/HIV-2 pseudovirion transduction model. This effect appeared to result from an indirect inhibition of HIV-1 reverse transcriptase (RT), likely mediated by Vpx and its incorporation into HIV-1 pseudovirions. Building on these findings, we sought to investigate additional roles of this unique accessory protein

This study aimed to address these knowledge gaps by:

(i) Assessing the efficacy of INSTIs and lenacapavir against HIV-2; and investigating the effects of raltegravir on SARS-CoV-2`s activity

(ii) Evaluating the transcriptomic changes induced by Vpx on gene expression, cytokine profiles and HIV-1 Tat protein

(iii) Analyzing the preferred integration sites of HIV-2 and determine the interaction between LEDGF/p75 and HIV-2 integrase during integration

3. MATERIALS AND METHODS

3.1 Plasmids and Inhibitors

This study utilized a panel of second generation plasmids to generate pseudovirions and express viral proteins. For HIV-1, the packaging vector psPAX2, the VSV-G envelope vector pMDG, and the pWOX transfer vector encoding the mCherry reporter were used. HIV-2 pseudoviruses were produced using packaging HIV-2 CGP plasmid, envelope plasmid pMDG, and the CRU5SINCSW transfer vector, which was modified to express mCherry under a CMV promoter. The lentiviral plasmids for HIV-2 were kindly provided by Dr. Joseph P. Dougherty (Robert Wood Johnson Medical School, NJ, USA). Cloning efficacy and vector integrity were confirmed by restriction digestion, polymerase chain reaction (PCR), and transduction efficiency assays. Antiviral inhibitors used in the study; including raltegravir, dolutegravir, cabotegravir, elvitegravir, bictegravir, and lenacapavir, were purchased from MedChem Express (NJ, USA) and reconstituted in DMSO to final concentrations ranging from 1 nM to 1 μM.

3.2 Inhibition profiling of INSTIs against HIV-2 in vitro

A HIS- tagged plasmid pQE-60 was constructed containing an 894bp DNA fragment of the HIV-2 integrase and preceded by six histidine residues at the 5' end of the cloning site. The constructed plasmid was then transformed in competent *Escherichia coli* BL-21(DE3) culture cells expressing pET11a bacteria and restricted with BamH1 and Nde1 restriction enzymes. Bacterial cultures were grown in LB broth supplemented with ampicillin (100 μg/ml) until reaching an OD_{600} of approximately 0.7–0.8. Protein expression was induced with 1 mM IPTG at 37°C for 3–4 hours. Cells were harvested by centrifugation, lysed enzymatically with lysozyme, and mechanically disrupted using ultrasonic sonication. During lysis, the bacterial pellet was suspended in Buffer A (10 mM imidazole, 50 mM Tris-HCI, 10% glycerol, 20 mM 2-mercaptoethanol, 1 mM PMSF, 0.1 mM EDTA, pH 7.6). Affinity chromatography was employed for protein purification where the supernatant was loaded onto 1 ml HIS column pre-equilibrated with Buffer B (0.1mM EDTA, 50 mM Tris HCI, 20 mM 2-mercaptoethanol, l mM PMSF, 10% glycerol, 10 mM imidazole, 1M NaCl, 25 mM CHAPS, PH 7.6). Elution was done with 2 ml of Buffer C (20 mM 2-mercaptoethanol, 50 mM Tris-HCI, 0.1 mM EDTA, 10% glycerol, 1 mM PMSF, 25 mM CHAPS, 1 M NaCI, 100 mM imidazole, PH 7.6). Eluted samples

were collected at different concentration peaks and run on 16 % SDS-PAGE again to identify the purified proteins. Bradford assay determined the protein concentration.

To measure integrase enzymatic activity, a non-radioactive ELISA-based strand transfer assay was used. A biotin-labeled donor oligonucleotide corresponding to the HIV-1 U5 LTR was immobilized on streptavidin-coated 96-well plates. The purified HIV-2 integrase was added to the wells with a target DNA in the presence of INSTIs. Strand transfer was detected using HRP-conjugated anti-digoxigenin antibodies, followed by reaction with TMB substrate and absorbance measurement at 450 nm.

3.3 Inhibition Profiling of INSTIs against HIV-2 in Cell Culture

3.3.1 Cell viability test in the presence of inhibitors

Initially, the cytotoxicity of the inhibitors was evaluated in Jurkat cells. In 96-well plates, 25,000 Jurkat cells were cultured in complete RPMI medium (1% L-glutamine, 10% FBS and 1% penicillin-streptomycin). On the subsequent day, cells were treated to a range of serially diluted inhibitor concentrations and then incubated for two days. Afterward, an MTT assay to evaluate the viability of the treated cells was performed following the manufacturer's instructions (Thermo Fisher Scientific).

3.3.2 Production of pseudoviruses

To generate pseudovirions, HEK-293T cells were cultured in T-75 flasks with DMEM medium containing 1% L-glutamine, 10% FBS and 1% penicillin-streptomycin until they reached approximately 70% confluence equivalent to 5 to 6×10^6 cells. Transfection was performed using polyethylenimine (PEI) with 10 µg of each plasmid required for pseudoviruses production. Following 5-6 hours of incubation in serum-reduced (1% FBS) medium, the transfection mix was replaced with complete DMEM. Viral supernatants were collected over three days, filtered, and concentrated via ultracentrifugation. The resulting pseudovirions were resuspended in PBS, aliquoted, and stored at -70°C. Quantification was achieved by measuring reverse transcriptase (RT) activity using a colorimetric ELISA-based assay.

3.3.3 INSTIs Inhibition profiling assay

For inhibition profiling, 25,000 Jurkat cells were seeded in 48-well plates and incubated overnight in RPMI supplemented with 1% L-glutamine and 10% FBS. The following day, cells were treated with a range of inhibitor concentrations spanning from 1 nM to 1 µM for INSTIs in antibiotic-free medium. After a 3-hour incubation with the inhibitors, the cells were transduced with 4 ng of HIV-1 and HIV-2 pseudovirions per well, facilitated by 8 µg/ml polybrene to enhance transduction efficiency. Following a 48-hour incubation, cells were harvested, and mCherry fluorescence was measured using flow cytometry to assess transduction efficiency. Data from 5,000 cells per sample were analyzed using FlowJo software, and dose-response curves were generated in GraphPad Prism to calculate the IC_{50} values for each inhibitor.

3.4 Activity of INSTIs against SARS-CoV-2

In partnership with the Laboratory of Virology and Chemotherapy (Rega Institute, KU Leuven, Belgium), we evaluated the antiviral activity of INSTIs against SARS-CoV-2 using A549-Dual™ hACE2-TMPRSS2 and VeroE6-GFP cells (M. van Loock, Janssen Pharmaceuticals, Beerse, Belgium). VeroE6-GFP cells (25,000 per well) were seeded in 96-well plates and pre-treated overnight with raltegravir and 0.5 µM MDR1 inhibitor CP-100356. The next day, cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.001. GFP expression was measured on day 4 post-infection to quantify viral replication. Inhibition was calculated after background subtraction and normalization to untreated, uninfected controls. Cytotoxicity was assessed in uninfected, treated cells, and CC_{50} values were estimated via logarithmic interpolation. SARS-CoV-2 variants Omicron BA.2, B.1.1.7, and the GHB strain (isolated from a traveller returning from Wuhan) were used, with identity confirmed by RT-qPCR. All viral assays were conducted in BSL-3 and BSL-3+ facilities at the Rega Institute.

3.5 Evaluation of Lenacapavir's effects on HIV-2 in cell culture

3.5.1 Inhibition profiling of lenacapavir

25,000 Jurkat cells were plated in 48-well plates in RPMI medium enriched with 1% L-glutamine and 10% FBS. After 24-hour incubation, the cells were treated with a concentration range of lenacapavir (1 pM to 1 nM) in antibiotic-free medium and incubated for three hours at

37 °C. Following drug exposure, HIV-2 pseudotyped virions were added at a dosage equivalent to 4 ng of reverse transcriptase (RT) activity per well, along with 8 μg/ml polybrene to enhance viral entry. After 48 hours of incubation, the cells were harvested in PBS and analyzed for mCherry fluorescence via flow cytometry to quantify transduction efficiency.

3.5.2 Production of hybrid pseudoviruses in the presence of lenacapavir

To further examine the impact of lenacapavir on viral production, hybrid pseudoviruses were generated in the presence of the drug. HEK-293T cells were cultured and pre-treated with lenacapavir at concentrations of 1 nM, 10 nM, and 100 nM for three hours before transfection with HIV-1 and HIV-2 plasmids. Following a brief transfection period (5-6 hrs) in serum-reduced DMEM (1% FBS), the culture medium was replaced with complete DMEM containing the corresponding concentrations of lenacapavir. After a 48-hour incubation, viral supernatants were collected and concentrated using Amicon Ultra centrifugal filters. Viral yield was determined using an RT ELISA, and the resulting hybrid pseudoviruses were used for downstream infectivity assays

Finally, to assess lenacapavir's effect on the HIV-2 capsid and subsequent infectivity, Jurkat cells (25,000) were transduced with the hybrid virions under the same conditions as the earlier assays. After 48 hours, mCherry fluorescence was measured by flow cytometry to determine transduction efficiency.

3.6 Molecular docking of INSTIs and Lenacapavir

The HIV-2 integrase structure was modeled using the SIVrcm intasome as a template, incorporating INSTIs (bictegravir, dolutegravir, and cabotegravir) and a DNA substrate. The SIV DNA sequence was modified to match our HIV-2 sequence, and nucleotide changes were introduced using UCSF Chimera. Structural minimization employed the Amber16 force field, with Li/Merz Mg^{2+} parameters and the TIP3P water model. Bictegravir and cabotegravir were geometry-optimized using Gaussian16 with RESP charge calculations. Docking was performed with PLANTS, targeting subunit A of the integrase active site. The docking center was defined at the catalytic Mg^{2+} ions, with a 12 Å radius, and the lowest-energy binding pose was selected. For lenacapavir, the HIV-2 p26 capsid protein structure was modeled from its amino acid sequence, and ligand-protein structures were converted to pdbqt format using AutoDock Tools.

Comparative docking was guided by the HIV-1 p24 capsid binding interface near Asn57 (PDB ID: 7RHN).

3.7 Transcriptomic Changes Induced by Vpx

3.7.1 Transfection of THP-1 cells

To investigate the effects of HIV-2 Vpx protein on the cellular transcriptome, 500,000 THP-1 cells were transfected with a GFP-tagged Vpx expression plasmid (pcDNA3.1-Vpx-NeGFP), derived from the HIV-2 ROD strain and obtained from Genscript Biotech. A functionally restricted mutant version carrying K68A and R70A substitutions (pcDNA3.1-MutVpx-NeGFP) and a GFP-only construct (pcDNA3.1-NeGFP) were used as controls. THP-1 cells were cultured in RPMI complemented with serum without antibiotics and seeded into 6-well plates with Opti-MEM prior to transfection using Lipofectamine LTX and 5 µg of the relevant plasmids. Expression was validated via fluorescence microscopy and flow cytometry. RNA was extracted 16 hours post-transfection using TRIzol and all experiments were conducted in duplicates.

3.7.2 RNA data sequencing and analysis

For transcriptomic profiling, RNA integrity was assessed using the Agilent 2100 BioAnalyzer and only samples with an RNA integrity number above 7 were used for sequencing. Libraries were prepared with the Ultra II RNA Sample Prep Kit following poly-A selection, fragmentation, and cDNA synthesis. Single-end sequencing (75 cycles) was conducted on the Illumina NextSeq 500. Quality control and alignment were handled by FastQC, Bowtie 2, and MultiQC, while EBSeq was used for differential gene expression analysis. Comparisons were made across three conditions: GFP-only, wild-type Vpx, and mutant Vpx. Gene ontology analysis was performed with topGO, filtering significant genes using a p-value < 0.05. HIV-1 interaction partners and virus-related metadata were further explored. Visualization was done using R packages including ggplot2, ggrepel, GOplot, and formattable.

3.7.3 Effects of Vpx on the cytokine profile

THP-1 cells (500,000 per well) were seeded in triplicates in 6-well plates in RPMI medium containing 1% L-glutamine and 10% FBS. After 3-hour incubation at 37°C, cells were activated with 100 nM PMA for 1 hour. The medium was replaced, and cells were incubated for 24 hours

to promote adherence and differentiation, confirmed by light microscopy. Cells were transfected with 5 µg of pcDNA3.1-Vpx-NeGFP or pcDNA3.1-NeGFP (mock) using Lipofectamine LTX in Opti-MEM, followed by a 5-hour incubation and medium replacement. Appropriate controls were included. After 24 hours, supernatants were collected post-centrifugation for cytokine analysis. Levels of IL-1β, TGF-β, IFN-α, and IL-6 were measured by ELISA (BD Biosciences and PBL Assay Science), with IFN-α quantified using the TCM kit. Statistical analysis was performed using the Kruskal-Wallis test in GraphPad Prism v7.0.

3.7.4 Effects of Vpx on HIV-1 Tat

HEK-293T cells (1×10^6) were seeded in T-25 flasks with 5 ml of complete DMEM one day prior to transfection. Cells were first transfected with 5 µg of either pcDNA3.1-Vpx-NeGFP or the empty pcDNA3.1-NeGFP vector (mock) using PEI; PEI-only cells served as non-transfected controls. After 24 hours, a second transfection was performed using 5 µg of pcDNA1-Tat plasmid with PEI. Following 24-hours incubation, supernatants were discarded, and cells were rinsed with PBS, harvested, centrifuged, and lysed in 300 µl of lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 25 mM NaCl, 0.5% NP-40, 50 mM NaF, pH 7.4). Lysates were incubated on ice for 30 minutes with intermittent vortexing, then sonicated and centrifuged. Protein concentration was measured using the Pierce BCA assay. Equal amounts of protein (15 µg per sample) were separated by 18% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with primary antibodies against HIV-1 Tat and β-actin, followed by HRP-conjugated secondary antibodies. Detection was done using Western Bright PICO substrate and visualized on an Azure600 GelDoc system.

3.7.5 Effects of Vpx on gene expression (RT-qPCR)

For infection assays, three primary HIV-2 subtypes A isolates (1010, 1654, and 1806) from West African patients, along with HIV-1 IIIB and BaL strains as controls, were propagated in PHA-stimulated PBMCs from healthy donors. Fresh PBMCs were added weekly, and supernatants were collected on days 7, 14, and 21, then stored at –80°C. THP-1 cells (250,000 per well) were plated in quadruplicates, activated with 100 nM Phorbol 12-myristate 13-acetate (PMA), and incubated for 24 hours to induce macrophage differentiation (confirmed microscopically). Cells

were then infected with 25 ng capsid-equivalent viral input and incubated for 48 hours. Reverse transcriptase activity was measured using a SYBR Green I-based PERT assay to confirm infection.

To assess Vpx-induced transcriptional changes, total RNA was extracted using the RNeasy Mini Kit. cDNA synthesis was performed using Superscript IV, an R1 primer, and RiboLock RNase inhibitor. RT-qPCR was carried out with SYBR Select Master Mix and gene-specific primers on a CFX96 Touch Real-Time PCR system. Relative expression levels were calculated via the ΔΔCt method, and significance was determined using the Kruskal-Wallis test in GraphPad Prism 7.0

In parallel, a transfection-based assay was conducted. THP-1 cells (500,000 per well) were transfected in triplicate with 5 µg of pcDNA3.1-Vpx-NeGFP, pcDNA3.1-MutVpx-NeGFP, or pcDNA3.1-NeGFP using Lipofectamine. After 16 hours, RNA was extracted with TRIzol and analyzed using the Cells-to-CT TaqMan kit with assays targeting SKOR2, U2AF1, CASP3, and GAPDH. RT-qPCR was performed under standard conditions, and data were analyzed using the ΔΔCt method. Statistical comparisons were made using a non-parametric t-test in GraphPad Prism 7.0.

Finally, caspase-3 activity, an indicator of apoptosis, was measured in THP-1 cells transfected with Vpx constructs. THP-1 cells (1×10^5 per well) were seeded in triplicate in 6-well plates using antibiotic-free RPMI medium with 10% FBS and 1% L-glutamine. Cells were transfected with 5 µg of pcDNA3.1-Vpx-NeGFP, pcDNA3.1-NeGFP, or pcDNA3.1-Mut-NeGFP using Lipofectamine LTX. Negative controls received Lipofectamine only, while positive controls were treated with 1 µM staurosporine to induce apoptosis. After 16 hours of incubation at 37 °C, cells were lysed in a buffer containing PMSF, DTT, Tris, and EDTA. Protein concentrations were quantified by BCA following centrifugation. Following cell lysis and protein quantification by BCA, caspase-3 enzymatic activity was assessed incubating 40 µg of lysate with 0.2 mM Ac-DEVD-pNA substrate in a colorimetric assay in a 96-well plate at 37 °C for 1 hour. Absorbance at 405 nm was recorded using a Synergy H1 microplate reader after 1-hour incubation at 37°C.

3.8 LEDGF/p75 – Integrase interaction Experiments

3.8.1 Confirmation of LTR sequences by PCR

To confirm successful integration of the pseudoviruses, 35,000 Jurkat cells were plated in full RPMI medium (10% FBS and 1% L-glutamine) without antibiotic and transduced with HIV-1 and HIV-2 pseudoviruses at a MOI of 1 in presence of 8 μg/ml polybrene to enhance viral entry. After 48 hrs incubation at 37°C, genomic DNA was extracted from transduced cells and subjected to PCR targeting HIV-1 and HIV-2 LTR regions. DNA was first fragmented via sonication for 2 minutes and 100 ng of the sheared DNA was amplified using Thermo Scientific Phusion High-Fidelity Master Mix and specific forward/reverse primers for the LTRs of HIV-1 (pWOX) and HIV-2 (CGW) vectors. The PCR program included an initial denaturation at 98°C for 30 seconds, followed by 35 cycles of 30-second extensions at 72°C, and a final extension for 10 minutes at 72°C. Amplified products were analyzed via agarose gel electrophoresis to visualize LTR-specific bands.

3.8.2 Detection of HIV-2 Integrase and LEDGF/p75 expression

For Integrase detection, Jurkat and HEK-293T cells (1×10^5 per well) were cultured in 24-well plates using complete RPMI and DMEM media, respectively, both supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. After incubation overnight, cells were transduced with 20 ng of HIV-2 pseudoviruses in the presence of 8 μg/ml polybrene. After 6 hours, cells were washed in PBS and lysed in buffer containing 50 mM Tris, 319 mM KCl, 33 mM $MgCl_2$, and 11 mM DTT (pH 7.8). Lysis was carried out on ice with intermittent vortexing every 10 minutes, followed by sonication (2 minutes total with 20-second pauses). Lysates were clarified by centrifugation (14,000 × g, 30 minutes, 4°C), and total protein concentrations were quantified using the Pierce BCA Protein Assay Kit. Equal amounts (30 μg) of total protein were loaded onto 14% SDS-PAGE gels for immunoblotting.

To examine the expression of LEDGF/p75, Jurkat and HEK-293T cells (5×10^5 per well) were cultured in six-well plates using complete RPMI and DMEM, respectively. After 24 hours, cells were washed with PBS and lysed in buffer containing 1 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 0.5% NP-40, 50 mM Tris-HCl, and 1 mM PMSF (pH 8.0).

Cell lysates were incubated on ice for 30 minutes with occasional vortexing. Lysates were then sonicated for 2 minutes with short intervals, centrifuged at 14,000xg for 30 minutes at 4°C, and the supernatants collected for protein quantification by bicinchoninic acid (BCA) assay.

For immunodetection, 40 µg of total protein per sample was resolved on 14% SDS-acrylamide gels and transferred onto nitrocellulose membranes. LEDGF/p75 was detected using a monoclonal anti-PSIP1 primary antibody (Sigma-Aldrich) and anti-mouse HRP-conjugated secondary antibody. HIV-2 integrase was detected using an anti-integrase antibody (NIH AIDS Reagent Program) and anti-rabbit secondary antibody. Signal development was achieved using Western Bright Femto or Pico chemiluminescent substrates, and imaging was done with the Azure 600 Gel Documentation System. B-actin was used as a loading control.

3.8.3 Proximity Ligation Assay (PLA) for LEDGF/p75–Integrase Interaction

Jurkat cells (2×10^4/well) were seeded in 96-well plates in RPMI full medium and transduced with HIV-2 pseudoviruses at a MOI of 1 in the presence of 8 µg/ml polybrene. Following centrifugation to enhance infection, cells were incubated at 37°C for different time points ranging from 0 to 12 hours (0h, 4h, 5h, 6h, 8h, 10h, and 12h). Afterward, cells were fixed with 5% paraformaldehyde and permeabilized using 0.3% Triton X-100. Duolink® in situ PLA was performed following the manufacturer's protocol using anti-LEDGF/p75 (1 µg/ml) and anti-HIV-2 integrase (1:2000) antibodies. Interaction signals were visualized using fluorescence microscopy (EVOS FLoid) at 100x magnification.

3.8.4 Effects of LEDGF/p75 silencing on the interaction with HIV-2 integrase

To assess the effects of LEDGF/p75 silencing, Jurkat cells (1×10^5/well) were transfected with 1.5 µg PSIP1-specific siRNA using Lipofectamine RNAiMax. After 24 hours, cells were lysed in 100 µl lysis buffer (1mM EDTA, 150 mM NaCl, 0.5% Deoxycholic acid, 50mM Tris, 1mM PMSF, 0.1% sodium dodecylsulfate (SDS) and 0.5% NP-40 pH 8.0). Lysates were incubated on ice for 30 minutes with intermittent vortexing for 10 seconds, sonication for 2 minutes, and centrifugation. Protein concentrations were determined using the Pierce BCA assay, and silencing was confirmed by western blot. For the proximity ligation assay, 20,000 siRNA-

transfected cells were plated in 96-well plates and transduced with HIV-2 pseudoviruses at MOI 1 in the presence of 8 µg/ml polybrene. Unsilenced cells served as controls. After 6 hours of incubation, cells were fixed with 5% paraformaldehyde, permeabilized with 0.3% Triton X-100, and subjected to the Duolink PLA protocol following the manufacturer's instructions.

3.9 Integration Site Analysis

Jurkat cells (35,000 per well) were seeded in 48-well plates in triplicate and immediately transduced with HIV-1 or HIV-2 pseudoviruses at a multiplicity of infection (MOI) of 1, using 8 µg/ml polybrene to enhance infection. The plates were centrifuged for 30 minutes and incubated for 48 hours at 37°C in a 5% CO_2 incubator. Following incubation, cells were washed with PBS and centrifuged, and the resulting pellets were resuspended in 200 µl PBS.

Genomic DNA was extracted from the transduced cells using the Invitrogen PureLink™ Genomic DNA Mini Kit, following the manufacturer's instructions. The protocol involved enzymatic digestion with Proteinase K and RNase A, lysis with buffer and ethanol, and purification through spin-column chromatography. DNA was eluted in 25 µl elution buffer, quantified using a Nano Drop 2000 spectrophotometer, and stored at –20°C prior to sequencing.

For integration site analysis, 1 µg of genomic DNA from five biological replicates per condition was used for library preparation with the NEBNext DNA Library Prep Kit. DNA was fragmented to ~350 bp by ultra sonication, ligated to Illumina-compatible adapters, and PCR-amplified. Purified libraries were quantified and size-assessed using the Agilent 2100 Bioanalyzer and sequenced on an Illumina NovaSeq 6000 platform (150 bp paired-end reads).

Raw reads were quality-trimmed with Trimmomatic and assessed using FastQC. Cleaned reads were mapped to the human genome (hg19) and reference HIV-1 and HIV-2 genomes using BWA-MEM. Viral integration sites were identified using Virus-Clip by detecting host-virus junctions with soft-clipped reads. Sites supported by at least two reads and consistent orientation were annotated with GENCODE v41 using BEDTools. Downstream analyses were conducted in R (v4.3.1) using GenomicRanges, retaining only autosomal, annotated loci. To explore chromatin context, ATAC-seq data from Jurkat cells (SRA: SRR15931092, SRR15931093) were aligned and analyzed with MACS2 and DeepTools. Integration density around ±2 kb of each site

was visualized using EnrichedHeatmap and circlize, facilitating a comparative view of HIV-1 and HIV-2 integration within accessible chromatin regions.

4. RESULTS

4.1 Efficacy of INSTIs against HIV-2 in vitro and in cell culture

The inhibitory efficacy of INSTIs (raltegravir, dolutegravir, bictegravir and cabotegravir), was initially tested against purified HIV-2 integrase in vitro. Despite the established efficacy of these INSTIs against HIV-1 integrase, none showed appreciable inhibition of HIV-2 integrase at concentrations of up to 10 μM. Control assays confirmed that the HIV-2 integrase retained its catalytic activity. To test the activity of the enzyme, we utilized a commercially available kit designed for HIV-1 although the manufacturer stated that measurement of HIV-2 integrase activity was also possible.

In contrast to the in vitro results, cell-based assays provided a strikingly different outcome, demonstrating potent inhibition of HIV-2 pseudoviruses' replication by all tested INSTIs. The observed inhibitory activity was notable, with IC_{50} values in the low nanomolar range. Among the tested compounds, dolutegravir exhibited the highest potency ($IC_{50} = 0.9 \pm 0.4$ nM), followed by cabotegravir, bictegravir, elvitegravir, and raltegravir.

4.2 Effects of INSTIs on the activity of SARS-CoV-2

In light of recent research into the potential cross-activity of antivirals across different viral families, selected INSTIs were screened for activity against SARS-CoV-2 variants (Wuhan-1, B.1.1.7, and Omicron BA.2). None of the tested INSTIs (raltegravir, dolutegravir, cabotegravir) demonstrated significant antiviral activity against SARS-CoV-2 in Vero E6 and A549-hACE2-TMPRSS2 cells.

4.3 Efficacy of Lenacapavir against HIV-2

Interestingly, lenacapavir, a capsid inhibitor known for its strong binding affinity to the HIV-1 capsid, exhibited even greater potency, inhibiting HIV-2 at picomolar concentrations ($IC_{50} = 206.2 \pm 0.2$ pM). Lenacapavir was further assessed for its effects on HIV-2 virion production and infectivity. Pseudoviruses were generated in the presence of increasing concentrations of lenacapavir, and the resultant virions were used to infect Jurkat cells. Although reverse

transcriptase activity remained unaffected across treatments in comparison with the controls, the viral transduction efficiency was significantly reduced, indicating that the produced virions, while retaining enzymatic function, were structurally compromised.

4.4 Molecular docking analysis of INSTIs and lenacapavir

Molecular docking simulations were conducted using homology-modeled structures of HIV-2 integrase and the capsid protein to assess potential binding interactions with INSTIs and lenacapavir. The results showed that the selected INSTIs—dolutegravir, bictegravir, and cabotegravir—were predicted to bind effectively within the active site of HIV-2 integrase, interacting with key catalytic residues and the essential Mg^{2+} ions, in a manner analogous to their interactions with HIV-1 integrase. Additionally, lenacapavir displayed strong binding to the HIV-2 capsid protein (p26), mirroring its interaction with the HIV-1 capsid (p24), thereby confirming that the structural features of HIV-2's capsid are sufficiently conserved for effective antiviral targeting.

4.5 Effects of HIV-2 Vpx on the cellular transcriptome

Transcriptomic analysis of THP-1 monocytes transfected with wild-type or mutant Vpx constructs revealed profound changes in host gene expression. Vpx was found to significantly alter the expression of hundreds of genes, implicating it in the modulation of various cellular pathways. Notably, genes involved in apoptosis (CASP3), splicing (U2AF1L5), and cell-cell adhesion (PCDHGC4) were upregulated, while genes related to immune regulation (SKOR2) and splicing (U2AF1) were downregulated. On the other hand, the functionally restricted Vpx altered the expression of genes involved in epithelial and skin regeneration, upregulating keratin-related genes (*KRT5*, *KRT6A*). GO analysis of genes differentially expressed in response to wild-type Vpx identified 27 molecular functions, 28 cellular component groups, and 72 biological process categories. These genes were enriched in processes such as transcription elongation, spliceosomal snRNP assembly, type I interferon signaling, and antiviral defense. Specifically, Vpx-regulated genes were associated with RNA polymerase II elongation, DNA-templated transcription, nuclear speck organization, ubiquitin binding, and suppression of innate immune responses.

4.6 Effects of Vpx on the cytokine profile and HIV-1 Tat

Further investigation revealed that Vpx increased the secretion of pro-inflammatory cytokines, including TGF-β, IL-6, and IL-1β, while type I interferons remained largely unchanged. Additionally, co-transfection of HEK-293T cells with HIV-1 tat and either wild-type or mutant vpx plasmids showed that wild-type Vpx markedly reduced Tat protein expression, as determined by western blot. The mutant Vpx (K68A-R70A) exhibited a milder inhibitory effect on Tat expression.

4.7 Gene specific validation

Quantitative RT-PCR was employed to validate specific gene expression changes of SKOR2, U2AF1, and CASP3, that were differentially affected by Vpx. Analysis showed significant down regulation of U2AF1 in THP-1 cells transfected with either wild-type or mutant Vpx plasmids (****$p < 0.0001$). In contrast, SKOR2 and CASP3 mRNA levels remained largely unchanged under the same conditions. In HIV-2 transduced cells, variant 1806 significantly downregulated U2AF1, while variant 1654 notably altered CASP3 expression (*$p < 0.05$). SKOR2 expression was not affected by either variant. While GO analysis had indicated CASP3 upregulation, RT-qPCR showed its down regulation by the 1654 variant. To resolve this, CASP3 enzymatic activity was measured using a colorimetric assay. Results showed increased CASP3 activity in cells transfected with wild-type Vpx, whereas activity remained at control levels in cells expressing the mutant Vpx. , confirming the down regulation of U2AF1 by both wild-type and mutant Vpx. Interestingly, variant-specific modulation of CASP3 was also observed, with increased caspase 3 activity in wild-type Vpx-transfected cells, despite discrepancies in transcript levels.

4.8 Interaction between LEDGF/p75 and HIV-2 integrase

Successful viral transduction and integration were confirmed by PCR amplification of the HIV-1/HIV-2 LTR regions from genomic DNA of transduced cells. Gel electrophoresis revealed bands corresponding to the expected sizes: approximately 640 bp for HIV-1 LTR and 850 bp for HIV-2 LTR

To assess the expression of LEDGF/p75 and HIV-2 integrase, western blot analysis was performed. LEDGF/p75 was detected in lysates of HEK-293T and Jurkat cells using an anti-PSIP1 monoclonal antibody, identifying two isoforms of LEDGF: p52 and p75. For integrase detection, Jurkat cells were transduced with HIV-2 pseudoviruses, and a 32 kDa integrase protein band was detected 48 hours post-transduction using an anti-HIV integrase antibody

The time dependent proximity ligation assay demonstrated the interaction signal between LEDGF/p75 and HIV-2 integrase as early as 4hrs post-transduction with the peak interaction at 6 hour time-point. To further explore this interaction, LEDGF/p75 was silenced using siRNA in Jurkat cells. Cells were incubated for 24 to 72 hours post-transfection to determine the optimal knockdown period. Western blot analysis confirmed efficient silencing at 24 hours, which was selected as the standard time point for subsequent experiments. PLA performed in LEDGF/p75-depleted cells revealed a substantial reduction in interaction signals between LEDGF/p75 and HIV-2 integrase compared to untreated controls, indicating that the interaction is specific and dependent on the presence of LEDGF/p75.

4.9 Integration site preferences of HIV-1 and HIV-2

Genomic DNA from HIV-1 and HIV-2-transduced Jurkat cells was sequenced to identify integration site patterns. Both viruses predominantly integrated into intronic and intergenic regions. However, HIV-1 displayed a higher frequency of integration within exonic regions compared to HIV-2.

Chromosomal distribution analysis demonstrated that while both HIV-1 and HIV-2 integrated across all chromosomes, their preferences differed. HIV-1 integrations were more frequently detected in chromosomes 6, 12, 14, and 15, whereas HIV-2 showed preferential integration in chromosomes 13, 17, 20, and the Y chromosome, with these four collectively accounting for approximately 70% of all HIV-2 integration events.

To assess chromatin accessibility at integration sites, ATAC-seq signal intensity was analyzed. HIV-1 showed a sharp, localized peak in accessibility at integration sites, with heat map values reaching up to 250, indicative of insertion into open chromatin regions. This was represented by prominent white and red areas on the heat map. In contrast, HIV-2 integration sites displayed a

flatter average profile with lower peak intensity (~40), and the corresponding heat map was dominated by blue tones, suggesting integration into less accessible or more randomly distributed genomic areas.

5. DISCUSSION

5.1 Inhibition profiling of INSTIs and lenacapavir against HIV-2

This study evaluated the inhibitory potential of INSTIs alongside the capsid inhibitor lenacapavir against HIV-2. In the purified in vitro integrase assays, none of the INSTIs showed significant inhibition of HIV-2 integrase, even at high concentrations (10 μM), despite demonstrating efficacy against HIV-1 under the same conditions. This discrepancy may be due to conformational differences in the HIV-2 integrase protein or suboptimal substrate compatibility, given that the assay design was based on HIV-1-derived oligonucleotides. Although catalytic activity was confirmed in control reactions, HIV-2 integrase displayed lower enzymatic efficiency than HIV-1, possibly due to its hydrophobic nature and tendency to aggregate, as previously described.

In contrast, all five INSTIs showed potent antiviral activity against HIV-2 in cell-based assays, with IC_{50} values in the low nanomolar range. Raltegravir inhibited the HIV-2 ROD-based strain with an IC_{50} of 2.1 nM, in agreement with earlier studies using clinical isolates. Similarly, elvitegravir and dolutegravir showed strong inhibition, consistent with previous findings. Although less well studied in the context of HIV-2, bictegravir and cabotegravir also demonstrated potent antiviral activity with IC_{50} values of 1.8 nM and 2.0 nM, respectively. These findings confirm that second-generation INSTIs maintain high efficacy against HIV-2, as observed in clinical studies of HIV-1 and in limited reports on HIV-2.

We also examined the potential of INSTIs raltegravir, dolutegravir, and cabotegravir to inhibit SARS-CoV-2 variants. None of the compounds showed significant antiviral activity against the Wuhan-1, B.1.1.7, or Omicron BA.2 variants. This outcome aligns with in vivo studies, and highlights the limitations of INSTI repurposing for SARS-CoV-2 despite some earlier in vitro and in silico evidence suggesting potential interactions.

Lenacapavir exhibited strong inhibitory activity against HIV-2, with an IC_{50} of 206 pM, surpassing its performance against HIV-1 (IC_{50} = 399 pM). Prior studies in HIV-1–infected cells reported EC_{50} values between 50–314 pM, depending on the cell type. However, published data on HIV-2 remain limited, with only one study confirming activity against two isolates. Our results support lenacapavir's potent capsid-targeting mechanism, with functional assays showing reduced transduction efficiency without affecting reverse transcriptase activity—consistent with its mode of action via disruption of capsid assembly.

Molecular docking further validated the experimental results. Bictegravir, dolutegravir, and cabotegravir formed stable complexes within the active site of HIV-2 integrase, coordinating effectively with Mg^{2+} ions, in agreement with structural models of strand transfer inhibition. Similarly, lenacapavir demonstrated strong binding to conserved capsid domains in HIV-2, resembling its interactions within HIV-1 capsid structures.

5.2 Transcriptomic changes induced by Vpx

To explore additional functions of the HIV-2 accessory protein Vpx, we performed transcriptome analysis on THP-1 cells transfected with either wild-type or mutant GFP-tagged Vpx constructs, using next-generation sequencing. This approach identified substantial changes in the expression of hundreds of genes involved in diverse cellular functions including immune regulation, transcriptional processes, protein folding, DNA methylation, and mRNA splicing.

Among the genes upregulated by Vpx were PCDHGC4, CASP3, NOP58, and SNX14, while SKOR2, U2AF1 and CORO7-PAM16 were notably downregulated. Many of these genes are known or suspected to play roles in viral replication or in modulating host cellular responses. For example, PCDHGC4, part of the protocadherin family, is implicated in cell adhesion mechanisms that could facilitate viral spread between cells through pathways such as PYK2 and FAK signaling. CASP3, a central mediator of apoptosis, has been associated with HIV-related CD4+ T-cell death, and is influenced by viral proteins Vpr and Vpu. In our study, CASP3 transcripts increased following Vpx expression, although qPCR data showed down regulation after 48 hours in cells infected with HIV-2 isolate 1654. Enzymatic assays, however, confirmed elevated CASP3 activity in Vpx-expressing cells, suggesting complex regulation at the post-transcriptional level or compensatory cellular responses.

The suppression of CORO7-PAM16, a gene involved in Golgi apparatus organization and vesicle trafficking, could impair HIV assembly, as these processes are essential for viral particle formation. The decreased expression of SKOR2, a transcriptional repressor implicated in TGF-β/Smad signaling, may lead to enhanced TGF-β pathway activity by removing repression. This is supported by our observations of increased SMAD4 and TGFBR1 expression and elevated TGF-β1 levels in cell culture supernatants. TGF-β1 is linked to HIV latency, immune suppression, and apoptosis, consistent with findings in SIV and HIV-2 infections.

Furthermore, Vpx expression was associated with increased secretion of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α. These cytokines contribute to immune activation and chronic inflammation seen in HIV infection, although, Vpx did not increase the expression of type I interferon, indicating some sort of an immunomodulatory effect.

The splicing factor U2AF1 was significantly downregulated at both mRNA and protein levels in response to Vpx. Given U2AF1's crucial role in pre-mRNA splicing, including for HIV transcripts such Tat and Rev, its reduction likely hampers viral protein production and replication. Supporting this, we observed lower HIV-1 Tat protein and mRNA levels in cells expressing Vpx, aligning with previous reports linking Tat dysregulation to viral latency.

Additionally, MYD88, a key adaptor molecule in innate immune signaling that activates NF-κB, was downregulated. Since NF-κB is essential for HIV proviral transcription, its decreased activation may facilitate viral latency, though further investigation is warranted.

5.3 Integration site analysis and LEDGF/p75–Integrase interaction

To explore viral integration patterns, we transduced Jurkat cells with HIV-1 and HIV-2 pseudoviruses and sequenced the host genomic DNA. Our results revealed that both viruses predominantly integrated into intronic regions; however, HIV-1 exhibited a higher tendency to integrate into exons gene regions directly involved in protein synthesis. This may partly account for HIV-1's higher replication efficiency and greater virion output compared to HIV-2.

The two viruses also displayed distinct chromosomal preferences. HIV-1 integrations were enriched in chromosomes 6, 12, 14, and 15, which include gene clusters essential for immune

function. For instance, chromosome 6 hosts the HLA gene family, chromosome 12 contains genes regulating growth and signaling, and chromosome 14 is vital for T-cell activity. These findings may reflect HIV-1's capacity to trigger robust immune activation, a hallmark of its pathogenesis.

In contrast, HIV-2 showed a preference for chromosomes 13, 20, and the Y chromosome regions characterized by lower gene density and reduced transcriptional activity. Chromosome 13 has gene-poor domains, chromosome 20 houses regulatory elements, and the Y chromosome is largely heterochromatic. These integration preferences may contribute to HIV-2's lower replication rate and its tendency toward prolonged latency.

Chromatin accessibility was assessed using ATAC-seq data. HIV-1 integration sites were strongly associated with open chromatin, as evidenced by a pronounced peak in ATAC-seq signal and concentrated hotspots in heat map analysis. In contrast, HIV-2 integrations occurred in regions with lower chromatin accessibility, showing a broader and more diffuse ATAC-seq signal, suggesting a less selective integration pattern and potentially reduced transcriptional output.

Although both viruses integrate within repetitive elements, prior studies have shown that HIV-1 favors gene-rich, transcriptionally active regions with moderate enrichment in SINEs especially Alu elements; often located near active promoters. This preference is largely mediated by host factors such as LEDGF/p75, which directs the integration complex toward euchromatic sites.

While LEDGF/p75's role in HIV-1 integration is well established, its involvement in HIV-2's life-cycle remains underexplored. Using a proximity ligation assay, we detected physical interaction between LEDGF/p75 and HIV-2 integrase, with peak association observed six hours after infection. Silencing of LEDGF/p75 using siRNA for 24 hours significantly reduced this interaction signal, indicating a potential functional role. Follow-up experiments involving LEDGF/p75 knockdown followed by integration site mapping are planned to better understand how this host factor influences HIV-2's integration.

6. CONCLUSION

This study presents a comprehensive evaluation of all currently approved INSTIs against HIV-2, using a standardized ROD-based integrase sequence and uniform cell culture assays. By minimizing variables such as host factor influence and co-treatment effects, the work offers a consistent comparison of the inhibitor's efficacy. While limited data previously existed on the activity of cabotegravir, bictegravir, and lenacapavir against HIV-2, our findings contribute valuable evidence supporting their potent antiviral effects. Further investigation is needed to elucidate the molecular basis of the reduced in vitro susceptibility of purified HIV-2 integrase to these inhibitors. In addition, we experimentally confirmed that raltegravir does not exhibit notable antiviral activity against SARS-CoV-2, reinforcing the importance of experimental validation of in silico predictions.

Transcriptomic profiling of Vpx-transfected cells revealed that HIV-2 Vpx has extensive regulatory effects on host gene expression. Gene ontology analysis showed that Vpx modulates pathways involved in antiviral defense, splicing machinery, transcription elongation, and immune signaling. Vpx was also associated with elevated levels of pro-inflammatory cytokines and appeared to enhance TGF-β1/SMAD signaling, potentially through SKOR2 down regulation. Furthermore, we observed a reduction in HIV-1 Tat expression in the presence of Vpx, suggesting a role in transcriptional silencing and latency that merits further investigation.

Finally, our comparative analysis of integration site patterns between HIV-1 and HIV-2 highlighted clear differences in genomic targeting and chromatin accessibility. HIV-2 integrase was shown to interact with LEDGF/p75, although further studies are needed to elucidate the interaction mechanisms. These findings provide additional information to HIV-2 integration dynamics and host interactions, offering a foundation for future studies aimed at targeting viral latency and improving therapeutic strategies.

7. SUMMARY

This PhD research investigated key aspects of HIV-2 biology, focusing on integration events, drug susceptibility, and the functional roles of the Vpx protein. The primary aims were to evaluate the susceptibility of HIV-2 to lenacapavir and integrase strand transfer inhibitors (INSTIs), explore the transcriptomic effects of Vpx, and characterize HIV-2 integration patterns and interaction with the LEDGF/p75 host factor.

The study utilized a multifaceted methodological approach. In vitro assays were conducted using purified HIV-2 integrase to assess inhibitor efficacy, while cell-based assays using pseudoviruses determined antiviral activity. Transcriptomic changes induced by wild-type and mutant Vpx were analyzed through RNA sequencing and gene ontology analysis in THP-1 cells. Cytokine profiling and Western blotting were employed to investigate Vpx's effects on HIV-1 Tat expression and host immune responses. Integration site mapping was performed using next-generation sequencing of pseudovirus-transduced Jurkat cells, complemented by ATAC-seq analysis to assess chromatin accessibility. LEDGF/p75-IN interaction was evaluated via proximity ligation assay and silencing experiments.

Key findings include the observation that INSTIs exhibited potent antiviral effects against HIV-2 in cell culture despite a lack of inhibition in in vitro enzyme assays, suggesting a complex interaction influenced by host cell context. Lenacapavir also showed efficacy against HIV-2 in culture. Vpx was found to modulate host gene expression, enhance pro-inflammatory cytokine production, and suppress HIV-1 Tat expression, likely through down regulation of U2AF1. Integration analysis revealed that HIV-2 preferentially integrated into intronic and intergenic regions, with a broader chromosomal distribution and weaker association with open chromatin compared to HIV-1. The interaction between HIV-2 integrase and LEDGF/p75 was confirmed, implicating its role in HIV-2 integration targeting.

In conclusion, the study expands our understanding of HIV-2 integration and drug susceptibility. It highlights possible interactions between HIV-2 integrase and host factors, underscores the potential of lenacapavir and INSTIs in HIV-2 treatment, and unveils novel functions of Vpx in viral regulation and immune modulation. These findings contribute valuable insights for

therapeutic strategies targeting HIV-2 and inform future research into viral latency and integration dynamics.

8. NEW FINDINGS

Our research provides among the earliest experimental evidence that the novel capsid inhibitor, lenacapavir, is effective against HIV-2 in cell culture. This was further supported by in silico docking, which demonstrated successful binding of lenacapavir to the HIV-2 capsid supporting its potential role in therapeutic strategies for HIV-2.

Transcriptomic and functional analyses demonstrated that Vpx protein may be possibly linked to the down regulation of HIV-1 in dual HIV-1 and HIV-2 infections through the down regulation of U2AF1, a splicing factor important for the functioning of HIV-1 tat protein, offering a new perspective on viral interactions.

Our preliminary data showed that HIV-2 interacts with the host chromatin integration factor LEDGF/p75, while exhibiting distinct genomic integration patterns and a preference for more closed chromatin regions, which may contribute to its distinct biological behavior.

9. LIST OF PUBLICATIONS PREPARED BY KENEZY LIFE SCIENCE LIBRARY

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Candidate: Irene Wanjiru Kiarie
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. Szojka, Z., Kunkli, B., **Kiarie, I. W.**, Linkner, T. R., Al-Muffti, A. S., Ahmad, H., Benkő, S., Jansson, M., Tőzsér, J., Mahdi, M.: Transcriptomic Analysis Reveals Key Pathways Influenced by HIV-2 Vpx.
   Int. J. Mol. Sci. 26 (8), 1-21, 2025.
   DOI: http://dx.doi.org/10.3390/ijms26083460
   IF: 4.9 (2023)
2. **Kiarie, I. W.**, Hoffka, G., Laporte, M., Leyssen, P., Neyts, J., Tőzsér, J., Mahdi, M.: Efficacy of Integrase Strand Transfer Inhibitors and the Capsid Inhibitor Lenacapavir against HIV-2, and Exploring the Effect of Raltegravir on the Activity of SARS-CoV-2.
   Viruses-Basel. 16 (10), 1-15, 2024.
   DOI: http://dx.doi.org/10.3390/v16101607
   IF: 3.8 (2023)

List of other publications

3. Mahdi, M., **Kiarie, I. W.**, Mótyán, J. A., Hoffka, G., Al-Muffti, A. S., Tóth, A., Tőzsér, J.: Receptor Binding for the Entry Mechanisms of SARS-CoV-2: Insights from the Original Strain and Emerging Variants.
   Viruses-Basel. 17 (5), 1-25, 2025.
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