Kinase Inhibitors and Nucleoside Analogues as Novel Therapies to Inhibit HIV-1 or ZEBOV Replication

by

Stephen D. S. McCarthy

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Laboratory Medicine and Pathobiology
University of Toronto

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Doctor of Philosophy

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Abstract

Without a vaccine for Human Immunodeficiency Virus type 1 (HIV-1), or approved therapy for treating Zaire Ebolavirus (ZEBOV) infection, new means to treat either virus during acute infection are under intense investigation. Repurposing tyrosine kinase inhibitors of known specificity may not only inhibit HIV-1 replication, but also treat associated inflammation or neurocognitive disorders caused by chronic HIV-1 infection. Moreover, tyrosine kinase inhibitors may effectively treat other infections, including ZEBOV. In addition, established nucleoside/nucleotide analogues that effectively inhibit HIV-1 infection, could also be repurposed to inhibit ZEBOV replication.

In this work the role of two host cell kinases, cellular protooncogene SRC (c-SRC) and Protein Tyrosine Kinase 2 Beta (PTK2B), were found to have key roles during early HIV-1 replication in primary activated CD4+ T-cells \textit{ex vivo}. siRNA knockdown of either kinase increased intracellular reverse transcripts and decreased nuclear proviral integration, suggesting they act at the level of pre-integration complex (PIC) formation or PIC nuclear translocation. c-SRC siRNA knockdown consistently reduced p24 levels of IIIB(X4) and Ba-L(R5) infection, or luciferase
activity of HXB2(X4) or JR-FL(R5) recombinant viruses, prompting further drug inhibition studies of this kinase. Four c-SRC kinase inhibitors (dasatinib, saracatinib, KX2-391 and SRC Inhibitor-1) significantly reduced HXB2 and JR-FL infection in primary CD4\(^{+}\) T-cells. Thus, these potent c-SRC inhibitors should be further evaluated in humanized mouse models of HIV-1 infection.

During 2014-16, the Ebola outbreak in West Africa prompted us to rapidly assess whether conventional nucleoside analogs could inhibit \textit{in vitro} ZEBOV replication. Employing a new lifecycle model of ZEBOV infection in level 2 biocontainment, combinations of nucleoside analogues and interferons were found to synergistically inhibit ZEBOV replication. These included zidovudine, lamivudine and tenofovir, confirmed to show antiviral activity against fully infectious ZEBOV-GFP in level 4 biocontainment. Findings from this thesis provided the rationale for further preclinical development of nucleoside analogue combination treatments, and a phase II EVD trial evaluating recombinant interferon in Guinea. Pre-clinical results using c-SRC kinase inhibitors also suggest that this approach could also be effective in EVD.
Acknowledgments

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Services, who gave me a platform to advocate for blood donor equality at a national level. And I am deeply thankful of Dr. Logan and Elizabeth Cohen, who through courageous actions, reported potential alternative therapies to treat Liberian Ebola patients in 2014.

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<tbody>
<tr>
<td>(-)ssRNA</td>
<td>negative-sense single-stranded RNA</td>
</tr>
<tr>
<td>(+)ssRNA</td>
<td>positive-sense single-stranded RNA</td>
</tr>
<tr>
<td>1-LTR</td>
<td>One Long Terminal Repeat</td>
</tr>
<tr>
<td>2ΔΔCt</td>
<td>Two delta delta Ct, comparative gene expression method</td>
</tr>
<tr>
<td>2-LTR</td>
<td>Two Long Terminal Repeats</td>
</tr>
<tr>
<td>IIIB</td>
<td>formerly HTLV-III B/H9, X4-tropic HIV-1</td>
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<tr>
<td>3P</td>
<td>Triphosphate</td>
</tr>
<tr>
<td>3TC</td>
<td>lamivudine, epivir</td>
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<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
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<tr>
<td>α4β7</td>
<td>gut-specific homing integrin</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<td>ABL1</td>
<td>Abelson murine Leukemia viral oncogene homolog 1</td>
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<tr>
<td>ACK1</td>
<td>Activated CDC42 Kinase 1</td>
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<tr>
<td>ADV</td>
<td>Adenovirus</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
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<tr>
<td>AMPK</td>
<td>AMP-Activated Protein Kinase</td>
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<tr>
<td>ANP</td>
<td>Acyclic Nucleotide Phosphonate</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APOBEC3G</td>
<td>Apolipoprotein B mRNA-editing Enzyme, Catalytic polypeptide-like 3G</td>
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<tr>
<td>ARG</td>
<td>Abelson-Related Gene</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral Therapy</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>AZT</td>
<td>Azidothymidine, zidovudine, retrovir</td>
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<tr>
<td>Ba-L</td>
<td>Ba-Lung, R5-tropic HIV-1</td>
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<tr>
<td>BCR</td>
<td>B-Cell Receptor</td>
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<td>BCV</td>
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<td>Immucillin-A</td>
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<td>BDBV</td>
<td>Bundibugyo Ebolavirus</td>
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<tr>
<td>BID</td>
<td>Twice a Day</td>
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<tr>
<td>BLK</td>
<td>B Lymphocyte Kinase</td>
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<td>BMVEC</td>
<td>Brain Microvascular Endothelial Cells</td>
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<tr>
<td>bNAB</td>
<td>broadly Neutralizing Antibody</td>
</tr>
<tr>
<td>Bp</td>
<td>Base Pair</td>
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<tr>
<td>BSL</td>
<td>Biosafety Level</td>
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<tr>
<td>BVDV</td>
<td>Bovine Diarrhea Virus</td>
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<tr>
<td>CA</td>
<td>Capsid protein, p24</td>
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<td>CADK</td>
<td>Calcium-Dependent Tyrosine Kinase</td>
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<tr>
<td>cART</td>
<td>combination Antiretroviral Therapy</td>
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<td>CCL5</td>
<td>Chemokine (C-C motif) Ligand 5, RANTES</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>CCR5</td>
<td>Chemokine (C-C motif) Receptor 5</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<td>CDC</td>
<td>USA Centers for Disease Control and Prevention</td>
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<td>CDV</td>
<td>Cidofovir, CFV</td>
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<tr>
<td>cDNA</td>
<td>complementary Deoxyribonucleic Acid</td>
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<tr>
<td>ChAd3-EBO-Z</td>
<td>Chimpanzee Adenovirus type-3 vaccine vector expressing ZEBOV GP</td>
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<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>ChiP</td>
<td>Chromatin Immunoprecipitation</td>
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<td>CHK</td>
<td>C-terminal SRC kinase-Homologous Kinase</td>
</tr>
<tr>
<td>CI</td>
<td>Combination Index</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myelogenous Leukemia</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CNS</td>
<td>Central Nervous System</td>
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<td>Co-IP</td>
<td>Co-Immunoprecipitation</td>
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<td>CR3</td>
<td>Complement Receptor 3</td>
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<td>CSK</td>
<td>C-terminal SRC Kinase</td>
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<tr>
<td>c-SRC</td>
<td>cellular-SRC, proto-oncogene protein tyrosine kinase c-SRC, pp60^{c-SRC}</td>
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<td>CVID</td>
<td>Common Variable Immunodeficiency</td>
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<td>CXCR4</td>
<td>Chemokine (C-X-C motif) Receptor 4</td>
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<td>c-YES</td>
<td>cellular homolog of the Yamaguchi Sarcoma virus oncogene</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<td>DC-SIGN</td>
<td>Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin</td>
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<tr>
<td>DFP</td>
<td>4-amino substituted Diphenylfuropyrimidine</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-Inducing Signaling Complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbego’s Modified Eagle’s Medium</td>
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<td>Dimethyl sulfoxide</td>
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<td>DN</td>
<td>Dominant Negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DNAL4</td>
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<td>dNTP</td>
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<td>Diphosphate</td>
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<td>Dithiothreitol</td>
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<tr>
<td>DV</td>
<td>Dengue Virus</td>
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<tr>
<td>DYRK1A</td>
<td>Dual specificity tyrosine-phosphorylation-Regulated Kinase 1A</td>
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<tr>
<td>Early RT</td>
<td>Early Reverse Transcripts</td>
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<td>EBOV</td>
<td>Ebolavirus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>ELISA</td>
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<td>EMD</td>
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<td>Envelope</td>
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<td>Extracellular signal–Regulated Kinase</td>
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<td>ESCRT</td>
<td>Endosomal Sorting Complexes Required for Transport</td>
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<td>Ebola Treatment Center</td>
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<td>Enterovirus</td>
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<td>Ebola Virus Disease</td>
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<td>EYFP</td>
<td>Enhanced Yellow Fluorescent Protein</td>
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<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<td>FasR</td>
<td>Fas Receptor</td>
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<tr>
<td>FAT</td>
<td>Focal Adhesion Targeting domain</td>
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<td>FERM</td>
<td>Four point 1/Ezrin/Radixin/Moesin domain</td>
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<td>FFU</td>
<td>Focus-Forming Units</td>
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<td>FYN Binding protein</td>
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<td>FYN</td>
<td>FGR- and c-YES-related protein kinase</td>
</tr>
<tr>
<td>Gag</td>
<td>Group-specific antigen, polyprotein precursor Pr55</td>
</tr>
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Chapter 1: Introduction
1.1 HIV-1 and Ebola: Global Health Problems

Notwithstanding decades of research in Human Immunodeficiency Virus type 1 (HIV-1) vaccine design [1], prevention strategies to reduce transmission [2], or drug treatments to limit and control infection [3], there are more than 36.7 million people living with HIV-1 worldwide as of 2015, and 1.1 million Acquired Immunodeficiency Syndrome (AIDS)-related deaths caused by HIV-1 each year [4]. Another virus of global concern is Ebola virus (EBOV), which infected at least 28,616 people and caused 11,310 deaths during the 2014-16 Zaire Ebola virus (ZEBOV) outbreak in West Africa [5]. EBOV was discovered before HIV-1, yet there are no approved antiviral agents to treat those infected [6]. The HIV-1 pandemic and EBOV outbreaks in Africa in recent years have caused global shifts in how governments treat viral illnesses that disproportionately affect the world’s poor, people who face stigma, and live in locations with limited access to medications, trained medical staff or well-equipped hospitals. This has also prompted changes in preclinical drug discovery research, to ultimately find effective treatments that can prevent, cure or reduce chronic illnesses caused by either HIV-1 or ZEBOV, in novel ways that can be implemented in resource limited settings or during a humanitarian crisis.

While treating HIV-1 infection in patients with combination antiretroviral therapy (cART) is well tolerated and strongly suppresses patient viral load, these treatments are not without some limitations [3, 7]. In the absence of a functional or sterilizing cure, the estimated 75,500 Canadians living with HIV-1 require life-time adherence to medications, which present new challenges in reducing the rate of new HIV-1 infections [8]. Prolonged cART does not significantly reduce the latent viral reservoir [3, 9], and there can be long-term toxicity associated with sustained treatment with HIV-1 therapies [10-12]. In addition, some regimens can cause unwanted drug-drug interactions in HIV-1 patients with comorbidities [13]. For instance, the protease boosting agents ritonavir and cobicistat inhibit cytochrome P450-mediated metabolism, altering the pharmacokinetics of co-medications [14, 15]. Thus there is a strong incentive to discover novel therapeutics that can complement and improve current cART medications.
Comparing to cART treatment of HIV-1, approved antiviral agents for treating Ebola Viral Disease (EVD) are much further behind. Critical and supportive intensive care guidelines for those suffering from EVD were still being developed during the 2014-16 ZEBOV outbreak in West Africa, and continues to be an active area of research [16]. Critical care interventions included: oral hydration therapy, intravenous fluid management to maintain blood electrolyte balance, loperamide treatment for severe diarrhea, and pain relief [16]. Experimental drugs that inhibit ZEBOV infection in vitro and in animal models of infection, which were safe and well-tolerated in phase I clinical trials, became fast-tracked for emergency phase II/III trials that began in 2014-15 (summarized in Table 1.1) [17-20]. These included trials assessing a cocktail of three monoclonal antibodies called ZMapp [19], small-interfering RNA (siRNA) called TKM-Ebola [21], transfusion of convalescent plasma [20] or recombinant interferon supplementation [22]. Nucleoside analogues approved for treatment of other viruses that showed anti-ZEBOV activity in vitro or in animal models of infection, such as favipiravir or brincidofovir, were also considered for emergency phase II/III trials in West Africa [18, 23, 24]. Some of these clinical trials are ongoing, in particular the PREVAIL vaccine trial in Liberia. Variability in non-randomized trial design and limited patient enrollment produced modest support for any particular treatment as the standard for treating EVD, while others were terminated before efficacy could be assessed [25]. Success occurred with one vaccine trial, providing 100% protection of those enrolled [26]. However treatment options for those with acute or chronic EVD are much desired. Thus, continued preclinical research into novel ways to inhibit HIV-1 and ZEBOV replication is warranted.
Table 1.1: Experimental drugs, therapeutics and vaccines fast-tracked for emergency II/III Ebola clinical trials in 2014-2016.

<table>
<thead>
<tr>
<th>Medication</th>
<th>Description</th>
<th>Mechanism of Action</th>
<th>Test species and evidence</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>TKM-Ebola</td>
<td>Small interfering RNA (siRNA)</td>
<td>Targets ZEBOV mRNA: RNA pol L, matrix protein (VP24) and polymerase cofactor (VP35)</td>
<td>Protects cynomolgus macaques [21], Phase II trial showed therapeutic benefit [27].</td>
<td>Tekmira Pharmaceuticals (Canada)</td>
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<tr>
<td>Brincidofovir</td>
<td>Lipid-conjugate of cidofovir (nucleoside analogue)</td>
<td>Selectively inhibits ZEBOV RNA-dependent RNA polymerase L</td>
<td>In vitro activity [28]. Phase II trial terminated (low enrollment) [24].</td>
<td>Chimerix (USA)</td>
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<tr>
<td>Favipiravir</td>
<td>Pyrazine-carboxamide derivative T-705 (nucleoside analogue)</td>
<td>Selectively inhibits ZEBOV RNA-dependent RNA polymerase L</td>
<td>In vitro, mouse model of EVD [23, 29]. Phase II trial shows efficacy in early stages of EVD [18].</td>
<td>Toyama Chemical (Japan)</td>
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<tr>
<td>ZMapp</td>
<td>Cocktail of three monoclonal antibodies</td>
<td>Binds viral envelope GP$_{1,2}$</td>
<td>Protects cynomolgus macaques [30]. Phase II trial showed therapeutic effect, but below statistical threshold [19].</td>
<td>Mapp pharmaceutical (USA)</td>
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<td>Interferon (IFN) β-1a</td>
<td>AVONEX (recombinant interferon)</td>
<td>Promotes type IIFN response to counteract VP24 and VP35</td>
<td>Prolongs macaque survival [31]. Phase II trial showed therapeutic effect in small cohort [22].</td>
<td>Biogen Inc. with CIHR scientists (Canada)</td>
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Vaccine Candidates

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<tr>
<th>Medication</th>
<th>Description</th>
<th>Mechanism of Action</th>
<th>Test species and evidence</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>ChAd3-EBOV-Z</td>
<td>Non-replicating, recombinant chimpanzee, adenovirus type-3 vector + booster</td>
<td>Expressed the GP of ZEBOV</td>
<td>Protects cynomolgus macaques [32]. Phase II/III trial results pending.</td>
<td>GlaxoSmithKline (UK)</td>
</tr>
<tr>
<td>rVSV-ZEBOV-GP</td>
<td>Live-attenuated, recombinant, vesicular stomatitis virus</td>
<td>Expressed the GP of ZEBOV</td>
<td>Protects cynomolgus macaques [33]. Phase II/III trials showed 100% efficacy [26].</td>
<td>National Microbiology Laboratory (Canada)</td>
</tr>
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1.2 Human Immunodeficiency Virus (HIV)

1.2.1 HIV-1 Epidemiology, Transmission and Replication Cycle

HIV-1, the primary cause of AIDS, was first clinically observed in the United States in men who have sex with men (MSM) in June of 1981 [34]. Otherwise healthy men succumbed to pneumonia caused by *Pneumocystis carinii*, and developed the rare skin cancer Kaposi’s sarcoma (KS) [34]. It was soon discovered patients were vulnerable to a variety of opportunistic infections caused by bacteria, viruses and fungi [35], which were recognized as AIDS-defining illnesses, permitting early classification of disease progression. In 1983-84, Dr. Robert Gallo, Dr. Luc Montagnier and Francoise Barré-Sinoussi were the first to report a new human T-lymphotrophic retrovirus from AIDS patients [36, 37]. Shortly thereafter, Dr. Robert Gallo independently demonstrated this new retrovirus to be the causative agent of AIDS [38, 39].

It has since been determined the HIV-1 pandemic started many decades prior to the first AIDS cases identified in North America. Human-to-human transmission in Africa occurred as early as the 1920’s in Kinshasa, the capital city of the Democratic Republic of the Congo (see Figure 1.1A, originally published in [4]). Early spread of HIV-1 from that era shares genetic similarity with simian immunodeficiency viruses (SIVs) that infect the common chimpanzee *Pan troglodytes*, suggesting HIV-1 originated as a zoonose from at least three separate cross-species transmissions to humans [40]. Another serotype, HIV-2, is found predominantly in West Africa and is associated with weaker transmission and less likely to cause AIDS, however co-infection with HIV-1 can complicate antiretroviral treatment [41].

HIV-1 is found in a variety of bodily fluids, but is primarily transmitted in semen, vaginal secretions or rectal secretions during intercourse, through transfusions of untreated blood products, by reusing needles without sterilization, and by mother-to-child (vertical) transmission from mixing of maternal and child blood at birth, or from breastfeeding [42]. Infant exposure to maternal blood and fluids during childbirth is the most common route of vertical transmission. In Canada, of the estimated 75,500 people living with HIV-1, 21% are unaware of their diagnosis [8]. The 2014 incidence of new infections was 2,570, where MSM accounted for 54.3% of new
**Fig. 1.1: Global and national spread of HIV-1/AIDS.** (A) Adult prevalence of HIV-1 in 2015, based on UNAIDS country surveillance [4]. The highest prevalence of HIV-1 is in 8 southern countries of Sub-Saharan Africa. The star represents where HIV-1 originated in the Democratic Republic of the Congo [43]. (B) The four primary modes of HIV-1 transmission in Canada, as of 2014 [8]. (C) Declining global incidence of new HIV-1 infections or mortality due to AIDS per year [4].
HIV-1 infections, followed by heterosexual intercourse (18.7%), people who emigrated from HIV-endemic regions (13.9%), those of aboriginal ethnicity (10.8%) and injection drug users (10.5%) (Figure 1.1B, originally published in [8]). Despite successes in reducing the global levels of children born with HIV-1, HIV-1 testing of pregnant women, HIV-1 prevention strategies, and increasing access to life-saving HIV-1 antiretroviral therapy, the global rate of new infections remains persistently high at 2.1 million new infections in 2015, while HIV-1 mortality declines (Figure 1.1C, originally published in [4]).

HIV-1 is an enveloped retrovirus with a short 9.7 kb genome on positive sense, single-stranded RNA ((+)ssRNA) (see Figure 1.2, originally published in [44] and [45]). The viral genome contains three genomic regions (gag, pol and env) that encode genes that directly participate in creating new virions, and 6 regulatory genes (tat, rev, nef, vif, vpr, and vpu). Together they encode 19 distinct proteins [46]. The gag region encodes genes for the structural proteins p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC) and p6. The pol region encodes genes for three viral enzymes: two subunits (p66 and p51) of reverse transcriptase (RT), integrase (IN) and protease (PR), as well as p15 (RNase H). Finally, the env region encodes genes for the two subunits of the external viral envelope protein, glycoprotein 120 (gp120) and glycoprotein 41 (gp41) [46]. The HIV-1 genome also encodes accessory proteins that regulate the viral lifecycle and subvert immune responses to infection: negative regulatory factor (Nef), two splice variants of trans-activator of transcription (p16 and p14 Tat), regulator of expression of virion proteins (Rev), viral infectivity factor (Vif), viral protein u (Vpu), viral protein r (Vpr), and a fusion protein encoded by tat, env and rev (Tev) [46].

During viral attachment and entry of a CD4⁺ T-cell, surface gp120, existing as a trimer of gp120-gp41 heterodimers, binds to host CD4 [47]. Gp120 then binds a host chemokine co-receptor, primarily α-chemokine CXCR4 or β-chemokine CCR5. CXCR4 is highly expressed on CD4⁺ T-cells and CCR5 on the cell surface of macrophages, leading to the nomenclature of X4 viruses (T-tropic) and R5 viruses (M-tropic), of which R5 viruses are more prevalent during early HIV-1 infection of a person and also more likely to be transmitted through intercourse [48, 49]. Following co-receptor engagement, virions that productively infect T-cells undergo internalization by receptor-mediated endocytosis [50]. Within the endosome a conformational
Fig. 1.2: HIV-1 virion and genomic structure. (A) Schematic of a fully matured HIV-1 virion. Transmembrane glycoprotein trimers, comprised of heterodimers of gp120-gp41, protrude from the lipid membrane envelope [44]. Structural p17 Matrix (MA) protein associates with the membrane envelope. The conical viral core is made of p24 capsid (CA) protein that surrounds two strands of (+)ssRNA genome that interact with p7 nucleocapsid protein (NC), three enzymes for replication (reverse transcriptase (RT), integrase (IN), and protease (PR)), as well as viral accessory proteins (Vif, Vpr and Nef). (B) Structure of the HIV-1 DNA genome with flanking 5’ and 3’ long-terminal repeat (LTR) regions and three genomic regions (gag, pol and env) that encode 19 distinct proteins through alternative splicing and cleavage by PR [45].
change in the glycoprotein trimer exposes the fusion domain of gp41, causing insertion of the fusion peptide into the target T-cell membrane [51]. Inside the cytoplasm, the virus core makes use of fibrous actin remodeling to dock outside the nucleus and shed capsid protein [52], creating the reverse transcriptase complex (RTC) containing both viral and host proteins (Figure 1.3, originally published in [53]). Viral reverse transcriptase synthesizes linear, double-stranded copy DNA (ds cDNA) from the viral RNA genome, which binds integrase and other host factors to form the pre-integration complex (PIC) [54]. The PIC is actively transported through a nuclear pore and integrated into a host chromosome by reactions catalyzed by integrase and host DNA repair proteins [55]. Failed integration of the cDNA are circularized by non-homologous end joining, homologous recombination, or autointegration, leading to closed circular forms with one or two long-terminal repeats (LTR), called 1- or 2-LTR circles [56]. Integrated provirus behaves as a set of genes while 1- or 2-LTR circles are episomal DNA that may lead to preintegration latency, a potential source of latent viral infection [57]. Expression of the integrated virus is regulated by several host transcription factors that bind enhancer and promoter sequences in the viral 5’ LTR, as well as viral Tat that binds the Transactivation Response region (TAR) of nascent HIV-1 RNA transcripts in the nucleus, considerably increasing transcription of the viral genome [58]. Unspliced and singly-spliced viral mRNA transcripts are exported into the cytosol by Rev, then translated into proteins by the ribosome [59]. Separate from other viral proteins, Env glycoprotein precursor (gp160) undergoes glycosylation and proteolytic processing in the endoplasmic reticulum and trans-Golgi apparatus [60, 61]. Mature gp120-gp41 glycoproteins are then inserted into the host plasma membrane as non-covalently bound trimers. Virus assembly at the plasma membrane is directed predominantly by Gag polyprotein precursor (Pr55) [62] and the host actin cytoskeleton [63], leading to virions budding with encapsidated genomic (+)ssRNA [64], viral enzymes, and host proteins located on the viral membrane and in the virion itself [65]. Viral protease cleaves Gag and Pol polyproteins during the release of the virus particle, creating fully mature virions capable of infecting new cells [66].

Following sexual transmission, dendritic cells (DCs), the most potent antigen-presenting cells (APCs) of the adaptive immune system, play a key role in acquiring HIV-1 at mucosal surfaces and disseminating the virus during early infection [67, 68]. Binding of virus to C-type lectin
**Fig. 1.3: The HIV-1 replication cycle in T-cells.** HIV-1 gp120 binds to host CD4 receptor, and chemokine co-receptor CXCR4 or CCR5, which trigger lipid mixing and membrane fusion. The viral nucleocapsid core, now in the cytoplasm, initiates reverse transcription of proviral ds cDNA in the reverse transcriptase complex (RTC). The core uses actin filaments to dock outside the nuclear pore, where CA protein sheds and IN forms the pre-integration complex (PIC) with the cDNA. The PIC and host proteins transport the cDNA into the nucleus where it integrates into host DNA by IN, or host DNA repair enzymes circularize non-integrated cDNA into 1-LTR or 2-LTR circles. Integrated provirus can remain quiescent or be expressed by NF-κB or NFAT initiating transcription. Multi-spliced viral mRNA are produced until enough Tat and Rev protein cause unspliced mRNA to be synthesized and transported into the cytosol, forming the full-length HIV-1 RNA genome. Viral proteins assemble with genomic RNA and bud as immature virions, where PR activity cleaves proteins to create mature virions [53].
receptors on immature DCs, such as mannose receptor, langerin or Dendritic Cell-Specific Interacellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), leads to protection from degradation in endolysosomal compartments [69, 70]. There, HIV-1 can remain infectious for 1-3 days, providing enough time for immature DCs to migrate from the submucosa to local lymphoid tissues and efficiently infect CD4+ T-lymphocytes [71]. Transmission occurs via three routes: trans-infection through a virological synapse (VS) [72], exocytosis of virions from multivesicular bodies [73], or by cis-infection from budding progeny virus [74]. HIV-1 can also directly infect CD4+ T-cells at mucosal surfaces. In addition to CD4+ T-cells, HIV-1 also infects monocytes, macrophages and microglia cells [75], but predominantly infects and depletes the helper CD4+ T-cell population during the course of infection [76, 77]. In a matter of weeks, CD8+ cytotoxic T-cell and B-cell responses to control infection inadvertently provide immune pressure that selects for the evolution of HIV-1 quasispecies [78]. Given the extensive genetic diversity of HIV-1 in a host, virus expressing gp120 variants with different glycan shields can be selected to evade clearance by broadly neutralizing antibodies [79]. Escape from broad humoral responses results in persistent infection, causing depletion of Th17 CD4+ T-cells in gut mucosa [80], mucosal damage from pro-inflammatory cytokines released from activated T-cells, and loss of immune protection at intestinal mucosa [81]. Dysfunction at the intestinal barrier allows microbial translocation of bacteria may contribute to systemic immune activation [82], which is characteristic of chronic HIV-1 infection. This continued immune stimulation disrupts the dynamic regulation of CD4+ and CD8+ T-cells [83], leading to a gradual loss of peripheral CD4+ T-cells over time from persistent viral replication and apoptosis [84, 85].

Chronic immune activation and inflammation also causes lymphoid tissue fibrosis via regulatory T-cells (Treg) depositing collagen [86], and persistent antigen stimulation produces defective helper T-cells that are less responsive, leading to effector T-cell exhaustion over many years [87]. In addition, the immune system is unable to eliminate latently infected, central memory CD4+ T-cells that produce low copies of virus, despite a patient adhering to suppressive therapy and having an undetectable viral load [3]. Unfortunately, combination antiretroviral therapy (cART) is unable to prevent the homeostatic proliferation of latently infected CD4+ cells that harbor integrated provirus that are transcriptionally silent, nor access certain compartments of the
human body, such as the central nervous system, testes or gut-associated lymphoid tissue (GALT), where low levels of viruses are produced and HIV-1 RNA and DNA are detected [88-90]. These have been called anatomical sanctuaries, hypothesized to maintain the latent viral reservoir through low-level viral replication in resting CD4$^+$ T-cells, and are established within days of primary infection [91]. Integrase inhibitors have been demonstrated to reach viral sanctuaries such as the GALT, however they are unable to decrease the latent viral reservoir. Thus, designing new antiviral combination therapies to activate infected, quiescent T-cells, then clear infected cells after reactivation, has become a prominent area of HIV-1 cure research [92].
1.2.2 HIV-1 Treatments, Potential Vaccines, and New Therapeutics

In 1986-87, the first antiretroviral therapy (ART) approved for AIDS treatment in the United States was the nucleoside analogue zidovudine (AZT) [93-95]. AZT, an inhibitor of HIV-1 reverse transcriptase, was swiftly used to treat AIDS patients at high doses to increase the CD4+ T-cell population and reduce opportunistic infections, but was associated with acute and long-term drug toxicity such as anemia and neutropenia [96]. Moreover, drug resistance, as measured by HIV-1 antigen detected in serum, developed within 24 weeks of continuous AZT treatment [97]. It was also determined that HIV-1 contains an error-prone reverse transcriptase enzyme that introduces base substitutions, additions and deletions when it synthesizes double-stranded cDNA from the viral RNA genome [98]. These provide genetic variation in integrated provirus that are selected under pressure of drug monotherapy, leading to escape mutations and the evolution of drug resistant HIV-1 quasispecies [99-101]. In 1995, it was discovered that combining AZT with lamivudine (3TC) was superior at controlling viremia when compared to either monotherapy treatment [102]. This was superseded by triple drug therapies in 1996, which considerably suppressed patient viral loads [103, 104]. The drug strategy was termed highly active antiretroviral therapy (HAART), now called cART.

With 25 different single drugs and 14 fixed-dose combination regimens currently FDA-approved to treat HIV-1, the success of cART has turned HIV-1 infection into a chronic, manageable illness [7]. This paradigm shift has averted millions of AIDS-related deaths and controlled the rate of new infections in developed and developing nations with the highest HIV-1 burden [105]. First-line therapy for treatment-naïve adults include three drugs, consisting of two nucleoside reverse transcriptase inhibitor (NRTI) and an inhibitor from another drug class [106]. These include non-nucleoside reverse transcriptase inhibitors (NNRTI), inhibitors that target viral entry, fusion, integration or viral protease, and the potential inclusion of pharmacokinetic enhancers [106]. While cART regimens can be well-tolerated and have a low pill burden for patients, they require lifetime adherence to control viremia and any interruption can lead to rapid viral rebound in plasma [107]. Drug interactions, unwanted side-effects, and regiment adjustments can also lead to poor adherence among patients, which increase the risk of drug
resistance mutations that may cause virological failure of first-line cART [7]. The emergence of multi-drug resistant HIV-1 variants has prompted renewed focus into novel vaccine and therapeutic strategies to improve or replace cART [108], and ultimately end the AIDS pandemic by developing a functional or sterilizing cure [109, 110].

There have been numerous attempts over the last 30 years to design a safe, immunogenic, and effective vaccine to prevent or treat HIV-1 infection [1, 111]. These include active immunization strategies to elicit broadly neutralizing antibodies (bNAbs) [112], inducing CD8+ T-cell responses [113], priming with Adenovirus (ADV) or canarypox vectors [114], or passive immunotherapy with a human monoclonal antibody (mAb) [115]. After two decades of failed vaccine trials, and one trial that lead to an increase in HIV-1 acquisition [116], the 2004-2009 RV144 trial in Thailand became the first vaccine to demonstrate efficacy in preventing 60.5% of new HIV-1 infections after 1 year, and 31.2% after 3.5 years [1]. The prime-boost vaccine given to 16,402 participants was a combination of two strategies: a canarypox vector (ALVAC-HIV) expressing clade E env and clade B gag and pro, and recombinant gp120 from clade B/E (AIDSVAX). Participants that expressed IgG antibodies to the V2 loop of HIV-1 gp120 were the least likely to become infected [1]. Further immunogenicity testing of the vaccine was tested in South Africa in the 2013-14 phase I/II HVTN097 trial [117], providing the groundwork for the phase II/III HVTN702 trial recently initiated in 2016. Therapeutic vaccines to intensify cART treatment and limit establishment of the viral reservoir have produced few promising leads, with the exception of the VRC01 monoclonal antibody isolated from the B-cells of an elite controller [118]. Continued challenges remain in eliciting broadly neutralizing antibodies, maintaining durable cross-strain breadth, and improving B-cell and T-cell priming to develop an effective HIV-1 vaccine [108].

Other innovative strategies to prevent HIV-1 infection include male circumcision, preventative microbicides and pre-exposure prophylaxis (PrEP). Since 2008, Voluntary Medical Male Circumcision (VMMC) has successfully led to 11 million adolescent boy and adult male circumcisions in eastern and southern Africa, as part of the ongoing World Health Organization (WHO) strategy to prevent female-to-male HIV-1 infection [2]. To prevent male-to-female infection, a variety of HIV-1 microbicides are being developed (acidic buffers, surfactants,
polyanionic polymers, reverse transcriptase inhibitors, and other small molecule inhibitors), with different modes of delivery (gels, vaginal rings, tablets and nanoparticles) [119]. However microbicides derived from cART regimens raise concerns of potential selection of drug-resistant HIV-1 strains in women who seroconvert in microbicide trials [120]. In addition, cultural expectations, partner-related factors (ex. fear of disproval) and acceptability issues have so far led to low microbicide treatment adherence in clinical trials [121, 122]. In Canada, the recent expansion of PrEP is a promising new strategy to prevent new HIV-1 infections, complementing other prevention strategies such as access to HIV-1/STI testing and free condom distribution. PrEP involves administering two ART medications, often tenofovir disoproxil fumarate (TDF) with emtricitabine (FTC) in the single pill Truvada, to seronegative people who are at high risk of contracting HIV-1 from a sexual encounter or the sharing of needles [123]. This can inhibit HIV-1 before it disseminates from the site of infection at mucosal tissues, preventing systemic infection [124]. A recent randomized, double-blind trial called IPERGAY determined that when MSM participants were optimally adherent to PrEP, high plasma levels of antiretrovirals could prevent 86% of new HIV-1 transmissions [125].

In terms of pre-clinical HIV-1 cure research, two main approaches are gene-editing to remove integrated provirus, and latency reversal followed by clearing the viral reservoir. CRISPR/Cas9 technology is the leading gene-editing strategy to excise integrated HIV-1 provirus from the primary CD4+ T-cells of HIV-1 patients ex vivo [126]. However, insertions and deletions that follow Cas9 cleavage, created by host Non-Homologous End-Join (NHEJ) repair proteins, can either suppress HIV-1 replication or accelerate viral escape by selection of viral sequences refractory to Cas9 recognition [127]. Although a recent in vivo study has demonstrated proof-of-principle for removing HIV-1 viral DNA in transgenic mice with CRISPR/Cas9 [109], further research is needed to produce safe and efficient delivery of a mildly immunogenic viral vector amendable for clinical use. Lastly, a ‘shock and kill’ strategy to reactivate then clear latent HIV-1 infection, potentially curing a patient of HIV-1, involves pairing small-molecule Latency Reversing Agents (LRAs), such as Histone Deacetylase (HDAC) inhibitors, with immunotherapies that promote clearance of persistently infected cells [92]. Challenges remain in reversing latency in all cell types harboring integrated provirus [128], boosting HIV-1-specific
CD8⁺ T-cell responses in tissue sanctuaries [129], and combining multiple LRAs in a coordinated fashion with approaches to clear infection in animal models and human clinical trials [130]. These complimentary approaches to replace or improve conventional cART are promising areas of research. Yet there is also considerable interest in studying host T-cell factors that contribute to HIV-1 replication, as novel means to restrict infection and eliminate the viral reservoir of latently infected cells [131].
1.2.3 Host Kinases as Targets for HIV-1 Inhibition

Decades of basic HIV-1 research have revealed many complex interactions between HIV-1 and the host immune cells that it infects. The success of FDA-approved HIV-1 antivirals targeting host T-cell factors demonstrate both the critical nature of host proteins participating in the HIV-1 lifecycle, and their therapeutic value as components of cART [132, 133]. For example, Maraviroc (MVC) was developed as a selective CCR5 receptor antagonist to block binding of envelope gp120 [134]. Thus, discovering antivirals that target host factors essential for HIV-1 replication is a compelling area of research, as they may pose higher barriers to drug resistance by blocking multiple stages of infection, offer unique tissue distribution to purge the viral reservoir, or alleviate symptoms not targeted by conventional cART [135, 136].

With a genome encoding only 19 proteins, HIV-1 requires many host T-cell protein interactions in order to replicate. These interactions have been organized into protein networks in an HIV-1 human protein interaction database [137]. As many as 348 unique protein-protein interactions have been found between HIV-1 and human proteins in the Jurkat T-cell line [138]. Moreover, mutations caused by the error-prone HIV-1 reverse transcriptase are primarily selected to negate cellular restriction factors, exploiting host defense mechanisms that then improve viral fitness [139]. As with other enveloped viruses, HIV-1 promotes replication and avoids immune responses by modifying the host plasma membrane, which it also uses for its lipid shell during budding. For instance, viral Nef and Vpu modulate plasma membrane receptor expression and localization, to increase viral fitness and evade immune detection [140]. They can cause CD4 downregulation, which promotes viral egress by allowing newly synthesized gp120 to traffic to the cell membrane, and inhibit tetherin binding, which allows newly released particles to bud from the plasma membrane [140]. Alongside Vpu and Nef, HIV-1 encodes two other accessory proteins (Vif, and Vpr) to counteract host restriction factors, each playing unique roles in different T-cell types and at different stages of infection [141]. For example, host apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) is a cytidine deaminase packaged into newly synthesized virions, catalyzing the deamination of cytosine shortly after reverse transcription of nascent single-stranded viral cDNA in a new cell, dramatically impairing
Another host protein, SAM domain and HD domain-containing protein 1 (SAMHD1), can also interfere with reverse transcription by hydrolysing cytosolic deoxynucleoside triphosphates (dNTPs) [143]. Yet these host proteins are counteracted by Vif, which prevents APOBEC3G packaging into virions, and Vpx (the HIV-2 equivalent of Vpr), which mediates degradation of SAMHD1 [141]. Moreover, viral enzymes such as IN depend on host-cofactors to such an extent that drugs that interfere with integrase interactions with Lens Epithelium-Derived Growth Factor (LEDGF/p75), called LEDGIN’s, show pre-clinical promise as a new class of allosteric integrase inhibitors [144, 145]. Thus cellular proteins hijacked during the HIV-1 replication cycle continue to be attractive targets in developing new antiretroviral therapies.

In particular, HIV-1 modifies host signal transduction pathways that disrupt virtually every aspect of cellular metabolism [146]. When gp120 binds to the co-receptor CXCR4 or CCR5 during viral entry of a T-cell, the interaction also initiates signaling pathways downstream to promote intracellular viral replication post-fusion [147]. Corroborating this finding, CD4+ T-cells isolated from asymptomatic, HIV-1 infected patients show defective early tyrosine phosphorylation downstream of T-cell receptor stimulation [148, 149]. General inhibition of tyrosine kinase signaling with the broad-spectrum inhibitor genistein has demonstrated that tyrosine kinase signaling is essential for HIV-1 entry and intracellular steps shortly thereafter [150, 151]. It has been put forth that HIV-1 requires T-cell activation and reorganization of the cytoskeleton for productive HIV-1 entry and replication [152], altering tyrosine kinase pathways downstream of the T-cell Receptor (TCR) to facilitate its viral lifecycle. In support of this hypothesis, the addition of mitogenic stimuli can activate host kinases to permit HIV-1 replication in quiescent T-cells that is otherwise inefficient, further suggesting a potential role of host T-cell kinases in the HIV-1 lifecycle [153]. As mentioned earlier, chronic T-cell activation also has an important role in maintaining persistent HIV-1 infection, helper T-cell disregulation, and eventual exhaustion of effector T-cells [83, 87] further emphasizing the important roles of tyrosine kinase signal transduction.

Small lentiviruses, such as HIV-1, appear to have evolved to become phosphorylated by host T-cell kinases to facilitate infection in non-dividing cells, access the nuclear compartment, and
assemble proteins during viral egress [154]. All HIV-1 proteins are phosphorylated throughout intracellular viral replication, however the functional purpose for these modifications, and the kinases that regulate them, is an active area of research [155]. Thus far, phosphorylation events have been found to regulate: HIV-1 viral entry [147], actin remodeling [52, 135], reverse transcription [156], capsid shedding [157], nuclear translocation of HIV-1 ds cDNA [154, 155, 158], viral integration [159], post-integration repair and circularization of un-integrated cDNA [160], proviral gene transcription [161], mRNA transport [162], viral assembly [63], and budding from the host T-cell [163]. One of the most well studied HIV-1 proteins that modify cellular tyrosine kinase activity is Nef, which has pleiotropic effects on cell signaling and strong binding affinity to the SRC family of kinases (SFKs) [164-167]. Nef preferentially activates SFK members LCK, HCK, LYN and c-SRC through allosteric displacement of intramolecular SRC homology 3 (SH3)-linker interactions [164], however its functional role in activating SFKs during HIV-1 replication is still under investigation, such as the SFK-Nef contribution to AIDS progression [168].

Currently there are 28 small-molecule kinase inhibitors approved by the US Food and Drug Administration for various cancer indications (Figure 1.4, originally published in [169] and [170]). Their improved specificity from first generation inhibitors and known inhibition of host kinases has made them ideal for other illnesses outside of cancer, such as pulmonary fibrosis [171], arthritis [172], and potentially viral infections [173]. Unfortunately, HIV-1 patients taking cART are at higher risk for non-AIDS defining cancers such as lung adenocarcinoma, Hodgkin’s lymphoma and anal cancer, when compared with the average population [174]. It has been suggested this increased risk in due to heightened immune activation and inflammation [175]. Repositioning safe and well-tolerated kinase inhibitors, with published safety and efficacy for various cancer indications, offers the potential for a new class of antivirals to inhibit HIV-1. In particular, tyrosine kinase inhibitors are attractive for these purposes because of
Fig. 1.4: FDA-approved kinase inhibitors. (A) The cancer and non-cancer indications of small-molecule kinase inhibitors as of 2015. Dasatinib, a dual ABL/SRC inhibitor that has shown antiviral activity, is highlighted in red [169]. (B) Chemical structure of dasatinib, with emphasis of its mode of binding to the adenine pocket and hydrophobic pocket of ABL kinase [170]. (C) Co-crystal structure of dasatinib (purple) in the Adenosine Triphosphate (ATP) pocket of ABL, interacting with key amino acids residues (green) through hydrogen bonding (red dashed lines) [170].
extensive research into their molecular interactions with host proteins, known off-targets, ease of administration, biodistribution, and pharmacokinetics within humans [169, 170]. Some of these inhibitors, such as imatinib, have been used successfully to treat Kaposi Sarcoma lesions and Chronic Myeloid Leukemia (CML) in HIV-1 patients on cART [176, 177]. Others have shown antiviral activity during virus replication, such as the dual SRC/ABL inhibitor dasatinib, restricting Dengue Virus (DV) replication in vitro [178]. Moreover, extended use of drugs that inhibit HIV-1 directly are prone to selection of drug-resistant variants, whereas small-molecule inhibitors targeting host factors may pose a greater mutational barrier, reducing the chance of selecting drug-resistant viral strains [152]. Thus novel kinase inhibitors that reduce inflammation, target cancer, and restrict HIV-1 replication, would be ideal drug candidates to improve cART regimens.

In the human genome there are 32 non-receptor tyrosine kinases (NRTKs) that catalyse the phosphorylation of tyrosine residues on protein substrates, grouped into 10 families [146]. NRTKs regulate cell processes essential to life such as cell signaling, growth, differentiation, motility, adhesion and cell death [179-183]. While NRTKs are generally appreciated for having central roles in cancer and chronic inflammation [184], they are increasingly being recognized for playing significant roles during viral infections. However, there are specific gaps of knowledge stalling FDA-approved tyrosine kinase inhibitors from being repurposed to treat HIV-1 in conjunction with cART. In the following three sections, the role of SRC family kinases and focal adhesion kinases (FAK) during HIV-1 infection will be reviewed.
1.2.4 The SRC Family of Non-Receptor Tyrosine Kinases in HIV-1 Infection

In 1911, Dr. Francis Peyton Rous observed that a virus in cell-free filtrate can cause fibrosarcoma cancer in domestic chickens [185], latter named the Rous Sarcoma Virus (RSV). For this discovery of tumor-inducing viruses, Dr. Rous was awarded the Nobel Prize in Physiology or Medicine in 1966. This soon led to the search for the oncogene responsible for transformation caused by retroviruses. Drs. John Michael Bishop and Harold Eliot Varmus demonstrated in 1976 that the viral oncogene responsible for avian sarcomas was originally acquired from normal avian cells [186]. The viral oncogene causing sarcomas was called v-SRC, to distinguish it from the cellular homolog c-SRC. Drs. Bishop and Varmus received the 1989 Nobel Prize in Physiology or Medicine for their work showing the cellular origin of retroviral oncogenes, and that a malignant tumor can originate from normal genes that regulate growth within a cell. Then at the University of British Columbia in 1981, while investigating Fujinami Sarcoma Virus (FSV) regulation of v-FPS signaling, Dr. Anthony James Pawson made an important breakthrough as he uncovered the first modular domain that controls cellular signal transduction [187]. He identified a phospho-tyrosine binding site similar to a non-catalytic region of v-SRC, called the SRC homology 2 or SH2 domain, which became prototypic of other non-catalytic modules that modify kinase activity allosterically, or through binding to other signaling proteins [188]. Since these pioneering discoveries, hundreds of modular protein domains have been identified that underpin multiprotein signaling complexes. Much has also been learned of the structure, regulation, localization and function of v-SRC, c-SRC, and the related SRC family of non-receptor tyrosine kinases [189].

In humans, there are eight SFK members: c-SRC, LCK, FYN, HCK, LYN, FGR, c-YES and BLK [189]. The 52-62 kDa proteins play essential roles in the signaling of a variety of cell processes, such as proliferation, differentiation, motility, adhesion, and proper functioning of adaptive and innate immunity [189-191]. c-SRC, FYN and c-YES show ubiquitous expression in many cell types and tissues, while the remaining five have time-specific and cell lineage-specific expression, such as in hematopoietic cells [192-196]. SFKs are activated by the stimulation of a variety of transmembrane G-protein coupled receptors (GPCRs), such as the C-
X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 5 (CCR5), linking surface receptors with downstream signaling pathways such as Mitogen-Activated Protein Kinase (MAPK) activation [197]. SFKs have seven conserved domains that regulate their function (see Fig. 1.5 for their domain structure, originally published in [198]). The N-terminus contains a 15 amino-acid peptide sequence that forms the SH4 domain, involved in post-translational lipid modifications of the SFK such as myristoylation or palmitoylation [199]. These modifications target SFKs towards membrane-associated signaling and adaptor protein complexes in various cell compartments, or lipid rafts positioned at the inner leaflet of the plasma membrane. The SH4 domain is followed by a unique region of 50-90 residues that is characteristic to each SFK [199]. This intrinsically disordered region (IDR) can encode sequences that promote cleavage of the SFK protein, phosphorylation of residues that add further specificity to kinase activity, and localization signals. After the IDR is an SH3 module that mediates intra- and intermolecular protein binding by recognizing the proline-rich PxxP consensus sequence. Next there is an SH2 domain capable of binding proteins with phosphorylated tyrosine in a pYEEI consensus sequence [199]. A short SH2-linker region containing proline-rich sequences follows the SH2 domain, a target region for SH3 binding. This linker region is followed by a large SH1 domain containing the catalytic site for substrate and ATP binding, and an activation loop where the trans- or autophosphorylation of a specific tyrosine residue fully activates the kinase [199]. Lastly, there is a short, negative regulatory C-terminus tail with a terminal tyrosine residue that when phosphorylated, binds the SH2 domain.

While healthy cells tightly regulate the active and inactive conformations of each SFK, viruses have been implicated in altering their expression, activity or cellular localization [178, 200]. These can impair innate or adaptive immune responses to clear viral infection, promote spread of the virus within tissues or between cells, prevent apoptotic cell death of infected cells, or directly assist in the viral replication cycle [201]. For instance, cellular SFKs facilitate the entry of the coxsackievirus, the RNA replication of Hepatitis C Virus (HCV) and DV, as well as assembly of West Nile Virus (WNV) [178, 201-203]. The multiple mechanisms by which the SRC-family of kinases facilitate or impede the infectious cycle of HIV-1 will be examined in this section, suggesting potential roles for c-SRC interactions with HIV-1, reviewed further in chapter 1.2.5.
**Fig. 1.5: Domain structure of chicken SRC-family kinases (SFKs).**  (A) Linear representation of the seven domains characteristic of all SFKs: C-terminus tail with a negative regulatory Tyr residue, kinase domain (SH1), proline-rich SH2 linker, a domain that binds phosphorylated Tyr (SH2), a domain that binds proline-rich motifs (SH3), a short unique region, and an N-terminus that can be post-translationally modified (SH4).  (B) Phosphorylated Tyr527/530 binds SH2, bringing the SH3 domain in close proximity of the SH2-linker, holding the SFK in an inactive conformation. Dephosphorylation of Tyr527/530 and phosphorylation of Tyr416/419 activate the enzyme.  (C) General mechanism of receptor-mediated SFK recruitment leading to full SFK activation [198].
LCK

Lymphocyte-specific protein tyrosine kinase (LCK) is expressed in B-cells, T-cells, natural killer cells, brain tissue and in the spleen [204-207]. It localizes to the plasma membrane and to pericentrosomal vesicles in the cell, interacting with the cytoplasmic tails of transmembrane receptors [208]. LCK non-covalently associates with CD4 or CD8, plays a major role in TCR-CD3 mediated T-cell activation, and is a downstream signaling molecule of the interleukin-2 (IL-2) receptor [209, 210]. In CD4-expressing T-cells, 50-96% of LCK is bound to the cytoplasmic tail of CD4 receptors, through interactions between the N-terminus of LCK and cysteine residues on CD4 [211]. Cross-linking of CD4 on the cell surface transiently increases the enzyme activity of LCK, and it is believed autophosphorylation of Tyr394 participates in this increased LCK activation [212]. In JCaM1 T-cells expressing a defective splice variant of LCK, cells are unable to respond to TCR-CD3 stimulation [213]. However, expression of wild type LCK restored TCR signaling, demonstrating the significance of LCK in normal TCR-CD3 signaling pathways [213].

In the CD4+ T-cells isolated from chronically infected HIV-1 patients, these cells show defective responses to CD3 activation, reduced proximal TCR tyrosine phosphorylation, and decreased levels of LCK expression [148]. Paradoxically, acute infection of Jurkat T-cells with HIV-1IIIB show a global increase in tyrosine phosphorylation within 30 minutes, including activation and phosphorylation of LCK [214]. Gp120/CD4/LCK complex signalling can induce NF-κB nuclear translocation and gene transcription [215]. These studies suggest nuanced and different effects of LCK throughout HIV-1 infection, which appears to be protective during early entry as LCK associates with CD4. For example, point-mutations of LCK at Cys20 and Cys23, which prevent LCK association with CD4, results in greater HIV-1 replication [209]. Moreover JCaM1.6 T-cells, expressing truncated LCK, are far more infectable than Jurkat 45 cells, which lack CD45 and express much higher LCK activity [209]. It has also been demonstrated that short-term exposure of gp120 can transiently enhance the autophosphorylation and activity of LCK, while long-term incubation of gp120 with CD4+ T-cells decreases overall expression of LCK [148, 216]. This longer gp120 exposure also correlates with complete dissociation of LCK from CD4, followed by downregulated surface expression of CD4 [217]. Furthermore, HIV-1 gp120 can block the migration of CXCR4-expressing T-cells to stromal cell-derived factor 1 alpha (SDF-
1α), which is mediated by CD4/LCK signaling and associated with cofilin phosphorylation [218]. The precursor protein, gp160, can also disrupt adhesion between CD4+ T-cells and B-cells by downregulating lymphocyte function-associated antigen-1 (LFA-1), in an LCK-dependent manner [219].

The use of a variety of techniques to overexpress or knockdown LCK, coupled with colocalization experiments, have revealed many of the mechanisms by which HIV-1 proteins, in particular Nef, Vpu and Tat, interact with LCK to modify its initial protective function in order to facilitate viral replication. In transgenic mice expressing HIV-1 Nef, the animals exhibit profound thymocyte depletion and CD4+ T-cell lymphopenia [220]. It has been found that Nef depletes the population of double-positive thymocytes and impairs lineage commitment towards single-positive CD4+ thymocytes, which was dependent on reduced LCK activity [220].

Expressing constitutively active LCK in the mice rescued CD4+ T-cell maturation, potentially through increased affinity of the TCR- Major Histocompatibility Complex (MHC) interaction [220]. HIV-1 Nef disrupts the anterograde transport of LCK to the plasma membrane within T-cells [167]. It does this by interfering with the vesicular transport of newly synthesized LCK to membrane-microdomains, a mechanism dependent on the SH4 membrane-anchor domain of LCK [167]. Nef also impairs the formation of the immunological synapse (IS) and early T-cell signaling, by retargeting the TCR and LCK for recycling in endosomal compartments (see Figure 1.6, originally published in [221]). It induces the internalization and endocytosis of surface CD4 in a manner dependent on LCK in lymphoid cells [222]. In addition, Nef relocates LCK to the trans-Golgi network, effectively decoupling downstream LCK kinase signaling from the surface TCR-CD3 complex [223]. In this way, Nef diminishes TCR responses to antigenic stimulation while rerouting LCK and other kinases to the trans-Golgi network, promoting T-cell survival through IL-2 production and enhancing viral spread of infected cells [223].

Similar to Nef, the viral accessory protein Vpu can also subvert the subcellular localization of LCK, implicating LCK with Vpu-mediated downregulation of cell surface receptors [224]. Nef also recruits a variety of proteins, including heterogeneous nuclear Ribonucleoprotein K (hnRNP-K) and LCK, into a signaling complex that increases Tat-mediated HIV-1 transcription [225].
Fig. 1.6: Nef recycling of LCK and TCR impairs formation of the immunological synapse.

(A) In resting T-cells, LCK and T-cell receptors (TCRs) continuously traffic through early endosomes. Upon TCR engagement with an antigen, polarized recycling targets TCRs and LCK to the immunological synapse, inducing T-cell activation. (B) During HIV-1 infection, viral Nef strongly retains LCK in recycling endosomes. TCRs also accumulate in the early endosome from reduced trafficking to the plasma membrane. Both of these effects lead to inefficient targeting of LCK or TCR to form an immunological synapse upon antigen stimulation [221].
interaction offers one potential explanation of how Nef enables high levels of viral replication in HIV-1 infected people. In addition, LCK has been shown to participate in Tat-mediated induction of NF-κB reporter gene expression in Jurkat T-cells [226].

LCK kinase activity also influences viral egress and cell-to-cell spread of HIV-1. Activated LCK directly interacts with Gag protein through its unique domain in infected cells, and facilitates viral assembly at the plasma membrane [227]. It was determined that palmitoylation of the unique domain of LCK was required for the successful release of HIV-1 virus-like particles [227]. In a phosphoproteome study of signaling pathways essential for virus cell-to-cell spread, LCK and other distal pathways of the TCR were activated and found to be essential for viral dissemination by the virological synapse [228]. Taken together, LCK initially promotes protection against HIV-1 infection downstream of CD4 and TCR-CD3 engagement during acute infection, becomes decoupled from CD4 and relocalized to the trans-Golgi complex where it facilitates downregulation of cell surface receptors, assists with viral egress and viral spreading, and becomes downregulated during chronic HIV-1 infection.

**FYN**

Similar to LCK, the FGR- and c-YES-related protein kinase (FYN) is involved in TCR-CD3 signaling in CD4+ and CD8+ T-cells, as well as IL-2 production [149, 229]. FYN also participates in Central Nervous System (CNS) myelination during neuronal development and the formation of a variety of cancers [230, 231]. FYN can be found in many cell types, although a unique isoform of FYN, called FYN-T, is expressed only in hematopoietic cells and contributes to TCR-induced calcium fluxes of antigen-stimulated T-cells [232]. It participates in integrin signalling, associates with FYN binding protein (FYB) to modulate IL-2 expression, and innate immune signaling through Toll-Like Receptors (TLRs) expressed on T-cells [233-235].

In the CD4+ and CD8+ T-cells from HIV-1 non-progressors, FYN is super-activated compared with cells collected from uninfected controls or HIV-1 patients with AIDS symptoms [149]. This points towards a potential protective mechanism associated with FYN kinase activity. Corroborating this finding, S1T cells, which lack LCK and FYN expression, are more infectable
by HIV-1 [209]. Similar to LCK, pre-treating cells with HIV-1 gp160 prevents CD3-mediated activation of CD4+ T-cells and FYN phosphorylation, demonstrating that gp160/CD4 signaling also deregulates the TCR-CD3 activation of FYN [236]. Also similar to LCK, acute HIV-1\textsubscript{IIIB} infection of Jurkat T-cells activates FYN activity in the first 30 minutes of infection [214]. Furthermore, FYN can activate the transcription of the HIV-1 promoter by activating four NF-κB DNA-binding proteins in Jurkat T-cells [237]. This activity required the SH2 domain of FYN [237]. Unlike LCK however, the effect of FYN during HIV-1 infection is not through an interaction with Nef. While Nef can bind the isolated SH3 domain of FYN \textit{in vitro}, it does not bind full-length FYN, nor has a direct effect on FYN kinase activity \textit{in vivo} [164].

During the HIV-1 lifecycle, FYN has been shown to directly interact with viral Vif and promote virion assembly and release [227, 238]. In the absence of Vif, activated FYN is able to phosphorylate the cytosine deaminase APOBEC3G, increasing the level of phosphorylated APOBEC3G becoming encapsidated into budding virus, which can restrict viral replication in the cytosol in a subsequent target T-cell [238]. However in the presence of Vif, Vif protein binds the SH3 domain of FYN, reducing FYN tyrosine kinase activity. Vif also reduces the autophosphorylation of FYN [238]. This reduced FYN activity leads to less phosphorylation of APOBEC3G packaged into virions, counteracting this natural HIV-1 resistance factor in successive infection cycles and promoting viral infectivity [238]. Interestingly, palmitoylated FYN can enhance the assembly and release of HIV-1 virus-like particles, in a similar manner as LCK [227]. This was also dependent on the N-terminal sequence of FYN, localizing FYN to sites of virus budding at the plasma membrane [227]. Thus FYN activity has multiple effects on HIV-1 during the viral replication cycle, with different signaling functions as HIV-1 enters the cell, initiates viral transcription, packages viral and host proteins into virions, and buds from an infected cell.

**HCK**

Hematopoietic cell kinase (HCK) is primarily expressed in B-lymphoid and myeloid cell lineages, such as promoncytic cells and monocyte-derived macrophages, and is not expressed in CD4\textsuperscript{+} T-cells [239]. In phagocytic cells, HCK mediates proinflammatory cytokine production,
Fc-gamma receptor 1 (FcγRI) signaling, phagocytosis, migration and cell spreading [240-242]. It also has important functions in the migration and degranulation of neutrophils [243].

In primary, naive B-cells, it has been found that HIV-1 gp120 signals through integrin α4β7, resulting in reduced proliferative responses that correlated with downregulated expression of HCK [244]. This may contribute to the delayed and ineffective humoral response after acute HIV-1 infection, as these primary B-cells treated with gp120 also increased expression of the immunosuppressive cytokine TGF-β1 [244]. After B-cells, HCK is most strongly expressed in monocytes/macrophages, which are important target T-cells of HIV-1 replication and maintenance of the viral reservoir. In monocyte-derived macrophages stimulated by macrophage colony-stimulating factor (M-CSF), high HCK expression correlated with increased HIV-1 infection, and reducing HCK expression with antisense oligonucleotides blocked viral replication [245]. Of all the SFKs, viral Nef most strongly binds HCK, and this interaction has been described in great detail [164]. Nef activates HCK in vitro and in primary macrophages, and the Nef PxxP motif is essential for Nef binding to the SH3 domain of HCK [164]. This implies that Nef activates SFKs through intramolecular displacement of SH3 binding to the SH2-linker. Interestingly, dephosphorylation of the negative regulatory tyrosine on the C-terminal tail, or displacement of the SH2 domain, are not required for Nef-mediated HCK activation, suggesting this method of SFK activation of an otherwise inactive kinase is unique to Nef [164]. This Nef-HCK interaction has spurred the structure-based design of novel HCK inhibitors that can reduce HIV-1 infection without causing cell cytotoxicity [246, 247]. In transgenic mice expressing mutant Nef lacking the PxxP motif, none of the mice developed an AIDS-like disease [168]. However, mice expressing wild type Nef and HCK knockout only showed delayed onset of the AIDS-like phenotype [168]. This suggests the SH3 binding ability of Nef is critical for developing severe AIDS-like disease, but that it depends on the interaction with a more essential factor that binds PxxP on Nef, perhaps another SFK such as c-SRC.

Nef-mediated activation of HCK has multiple affects on intracellular HIV-1 replication, surface receptor expression levels and cell motility. Similar to Nef-LCK interactions, HIV-1 Nef inhibits the anterograde transport of HCK to plasma membrane microdomains, causing HCK to accumulate at recycling endosomes and the trans-Golgi network [167]. Through HCK, Nef
perturbs the intracellular maturation and trafficking of receptors destined to the plasma membrane, such as CSF-1 receptor (CSF-1R) expression on macrophages [248]. M-CSF is a cytokine released during viral infection, and promotes the differentiation of macrophages. However during HIV-1 infection, Nef activation of HCK in the trans-Golgi network downregulates surface expression of mature CSF-1R by causing accumulation of under-N-glycosylated CSF-1R [248]. It was determined that Nef-activated HCK disrupts the distribution of Golgi Matrix protein 130 (GM130), which is required for efficient protein glycosylation [249]. Moreover, Nef-activated HCK induced ERK signaling that caused serine phosphorylation of the GM130-interacting protein Golgi Reassembly-Stacking Protein 1 (GORASP1), causing this structural protein to unstack Golgi cisternal membranes [249]. The Nef interaction with the SH3 domain of HCK is also responsible for signalling that downregulates MHC I surface expression, potentially enabling infected cells to evade killing by CD8+ cytotoxic T-cells [165]. In immature dendritic cells, HIV-1 Nef also induces the downregulation of CD1 surface receptors, a class of non-MHC lipid antigen-presenting proteins [250]. This effect was mediated by activated HCK and p21-activated kinase 2 (PAK2) [250]. The PxxP-SH3 interaction between Nef and HCK can also enhance the incorporation of HCK into budding viral particles from 293T cells [251]. In addition, Nef inhibits the amoeboid migration pattern of HIV-1 infected monocytes-derived macrophages, promoting instead mesenchymal migration in vitro [252]. Mesenchymal motility requires podosome regulation and extracellular proteolysis of the matrix, and it was found that Nef alters the stability, size and proteolytic function of podosomes through increased HCK activity and Wiskott-Aldrich Syndrome protein (WASp) [252]. This Nef-HCK-mediated migration reprogramming in macrophages could have important effects on the dissemination and spread of HIV-1 infection in vivo, as transgenic mice expressing Nef show increased tissue infiltration [252] and HIV-1 patient tissues accumulate macrophages during infection [253].

While Nef-induced activation of HCK facilitates HIV-1 infection, HCK kinase activity impairs later stages of virus assembly and release. This HCK activity is counteracted by viral Vif. Using living cell fluorescence microscopy, it has been observed that Vif multimerization, an indication of its role in Gag viral assembly and genomic RNA packaging into viral particles, is altered by
HCK expression in HeLa cells [254]. In HCK-expressing cells infected with Vif-deleted HIV-1, both the release of viral particles and subsequent virion infectivity are strongly reduced [255]. Similar to FYN, Vif has been shown to bind the SH3 domain of HCK, reducing its activity and preventing autophosphorylation of HCK tyrosine kinase [255]. Also like FYN, HCK phosphorylates tyrosine on APOBEC3G, increasing the levels of this restriction factor being packaged into budding virions [238]. As expected, HIV-1 expressing Vif can mitigate this by reducing HCK activity [238]. Thus, the opposing effects of Nef and Vif on HCK activity demonstrate the complexity of HIV-1 fine-tuning SFK activity, both in their location and timing, during the virus replication cycle.

**LYN**

LCK/c-YES novel tyrosine kinase (LYN) is primarily expressed in hematopoietic cells, neural cells, and liver tissue [256-258]. LYN kinase activity has important function in regulating degranulation of mast cells, erythrocyte differentiation, and cell activation [259]. In particular, LYN has been implicated downstream of B-Cell Receptor (BCR) engagement [260]. Activation of the BCR receptor leads to LYN phosphorylating Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) on the cytosolic portions of receptor proteins, recruiting and activating Spleen-associated tyrosine Kinase (SYK), Phospholipase C gamma 1 (PLC-γ1) and Phosphoinositide 3-Kinase (PI3K), which further transduce activation signals, induce Ca^{2+} mobilization, and promote B-cell proliferation and cell differentiation [194, 261, 262].

As with LCK, HCK and c-SRC, LYN has been shown to bind the Nef PxxP motif through its SH3 domain, causing allosteric displacement of the SH2-linker, which increases LYN kinase activity [164]. LYN and HCK share a key Ile residue in their SH3 domain, responsible for their high affinity binding to a hydrophobic pocket within the core of Nef [164]. However, a biological role for Nef activation of LYN during HIV-1 infection has yet to be described. Yet the interactions between LYN, HCK or c-SRC with Nef are highly conserved, as shown with different Nef proteins isolated from HIV-1 M-group subtypes [263]. In these experiments testing infection of CEM-T4 lymphoblast cells with chimeric HIV-1 expressing each Nef protein, Nef-SFK binding could be uniformly disrupted with the compound DFP [263]. This
demonstrates that Nef-dependent activation of SFKs are a conserved feature of M-group HIV-1 isolates.

Studies have also demonstrated other roles of LYN kinase activity during HIV-1 infection, with particular focus on alterations to hepatocytes and dendritic cells. Coinfection with HCV and HIV-1 has led to research into the effects of HIV-1 envelope proteins on hepatocytes, as patients with both viruses are more likely to develop liver disease or liver cirrhosis [257]. HIV-1 gp120 has been found to cause bystander apoptosis of uninfected hepatocytes through a Signal Transducer and Activator of Transcription 1 (STAT1) signaling pathway that involved activation of LYN, mitogen-activated protein kinase 14 (p38α), and Protein Kinase C (PKC) [257]. In immature monocyte-derived DCs, it has been demonstrated that inhibiting LYN with the SFK inhibitor PP2, or reducing LYN expression with targeted siRNA, leads to greater HIV-1 production when co-cultured with primary CD4+ T-cells [264]. This finding suggests LYN may play an important role in the transfer of HIV-1 from DCs, as LYN has previously been described to associate with the cytoplasmic tail of DC-SIGN [265]. Furthermore, LYN kinase activity has been implicated in immune evasion strategies of HIV-1 in immature DCs as they respond to free HIV-1 virions and complement-opsonized HIV-1 [266]. While exposure of immature DCs to free virus induced proinflammatory cytokines IL-1β, IL-6, and Tumor Necrosis Factor alpha (TNF-α), these were dampened in cells treated with complement-opsonized HIV-1 [266]. Instead, these immature DCs showed a different signaling pattern that involved the activation of IFN regulatory factor 3 (IRF3) and phosphorylated LYN, leading to enhanced infection of the immature DCs [266]. It is hypothesized that C3-opsonized HIV-1 could be signaling through TLRs and complement receptor 3 (CR3), which leads to a LYN/PI3K pathway that suppresses inflammatory responses in immature DCs [266]. These findings suggest HIV-1 could be subverting the host complement system in a LYN-dependent manner, which may have implications in immature DCs of the genital tract and rectal mucosa, as these are one of the first cell types to contact HIV-1 shortly after sexual transmission of the virus.

The effects of LYN kinase activity during HIV-1 infection have been most documented in monocytes and macrophages, downstream of CCR5 and CXCR4 receptor stimulation. Treating monocytes-derived macrophages with Macrophage Inflammatory Protein 1 Beta (MIP-1β) leads
to activating a CCR5/LYN/ERK-1/2 pathway, which can similarly be triggered by stimulation with HIV-1 gp120 [267]. This inappropriate macrophage activation by gp120 can induce the production of TNF-α and other proinflammatory cytokines, which could contribute to AIDS pathologies and HIV-1 associated dementia [267]. A separate study on primary human, monocytes-derived macrophages, also found that stimulation of CCR5 with gp120 or whole virions triggers IL-1β release, which depended on coupling of the Gaα subunit to the CCR5 receptor [268]. This gp120/CCR5 stimulation also led to concomitant PTK2B and PI3K activation, translocation of both of these signaling proteins from the cytoplasm to the plasma membrane, and formation of a signaling complex with activated LYN tyrosine kinase [268]. Monocytes migration is also regulated by LYN activity downstream of CXCR4 receptor engagement with its ligand, SDF-1α [269]. When Brain Microvascular Endothelial Cells (BMVEC) are activated with TNF-α, IL-1β or treated with HIV-1 gp120, it has been observed that SDF-1α-treated monocytes no longer adhere to Intercellular Adhesion Molecule 1 (ICAM-1) ligands, but instead become migratory [269]. Conversely, targeted siRNA knockdown of LYN prevented the SDF-1α-mediated migration of monocytes, promoting monocytes attachment to ICAM-1 on activated BMVECs [269]. This data strongly suggests that LYN kinase regulation of SDF-1α-activated monocytes migration could have an important role in the infiltration of monocytes into the CNS and past the blood-brain barrier, which may contribute to the various neurological disorders associated with chronic HIV-1 infection.

**FGR, c-YES and BLK**

For the final three SFK members, Feline Gardner-Rasheed tyrosine kinase (FGR), cellular homolog of the Yamaguchi sarcoma virus oncogene (c-YES) and B lymphocyte kinase (BLK), less is known about their roles during HIV-1 infection.

Expressed exclusively in hematopoietic cells, FGR is found in myeloid cells and B-cells, and localizes to plasma membrane ruffles [195]. It acts a negative regulator of cell migration and adhesion downstream of integrin beta-2 (ITGB2) signaling [270]. FGR overexpression has been associated with a subset of myeloid leukemia and ovarian tumors [271, 272]. Interestingly, the amino-acid sequence of FGR shares a limited but significant N-terminal sequence homology
with HIV-1 Nef [273]. Despite strong expression in macrophages and overlap in signaling functions with HCK, FGR does not bind or become activated by Nef, likely because FGR lacks the critical Ile residue in its SH3 domain [164]. In a study of the effects of HIV-1 gp120 on downstream signaling in naïve B-cells, it was determined that gp120 binds integrin α4β7, restricting proliferation [244]. This signaling was associated with increased production of the immunosuppressive cytokine Transforming Growth Factor Beta 1 (TGF-β1), increased expression of the inhibitory surface receptor FcRL4, and downregulation of FGR expression [244]. Thus, HIV-1 may be subverting early HIV-1-specific humoral immune responses by causing B-cell dysfunction through integrin signaling through FGR. Early HIV-1 infection also appears to alter FGR expression in vaginal epithelial cells. Compared with untreated control cells, vaginal epithelial cells treated with HIV-1 gp120 showed upregulation of FGR kinase expression, which occurred in concert with genes that promote inflammation and proteases that are capable of weakening the vaginal epithelium [274]. Accordingly, FGR may have unique signaling functions that are tissue specific during HIV-1 infection, reminiscent of how LCK and HCK exhibit up- and down-regulated activity at specific stages of the HIV-1 replication cycle.

In contrast with FGR, c-YES tyrosine kinase is ubiquitously expressed in many cells and tissues, having an essential role in regulating cell growth, cell survival, cell adhesion, cytoskeletal rearrangements, and cell differentiation [275-277]. c-YES signaling has important functions in glucose activation of the cell cycle, translocation of Epidermal Growth Factor Receptor (EGFR) into the nucleus during tumor progression, and Platelet-Derived Growth Factor Receptor (PDGFR) induced chemotaxis [278-280]. c-YES also has an important role in mediating chemokine-directed T-cell motility [281]. Jurkat T-cells treated with SDF-1α stimulates CXCR4, which induces c-YES to phosphorylate Collapsin Response Mediator Protein 2 (CRMP2) at Tyr479, leading to T-cell polarization and lymphocyte migration [281]. As with FGR, the SH3 domain of c-YES does not permit interactions with HIV-1 Nef [164]. In a study of HIV-1 Tat modifying global gene expression in primary CD4+ T-cells, it was found after 24 hrs of Tat exposure that c-YES showed a modest, yet significant reduction in gene expression [282]. This correlated with the CD4+ T-cells increasing secretion of 11 different cytokines, with notable production of IL-17, and proinflammatory gene expression reminiscent of the Th17 phenotype [282]. However,
this finding is incongruent with another study examining the role of c-YES and apoptosis-related genes during HIV-1 infection of Jurkat T-cells [283]. In this work, Jurkat T-cells infected with HIV-1 showed increased c-YES expression within 3 days, as confirmed by Western blot [283]. The authors suggest that c-YES may be signaling downstream of the Fas receptor (FasR), which can oligomerize and trigger apoptosis through the Death-Inducing Signaling Complex (DISC) [283].

Lastly, BLK is a tyrosine kinase expressed in pancreatic β cells and hematopoietic cells, with essential function in B-cells in particular [284, 285]. It participates in pre-BCR signaling, B-cell development, and B-cell differentiation [286, 287]. As with other SFKs, BLK transmits signals from cell surface receptors, and has been implicated in B-cell associated autoimmune disorders [288]. Little is known of changes to BLK activity in response to HIV-1 infection, although a BLK gene variant has been found to disrupt B-cell proliferation and the ability to elicit antigen-specific CD4+ T-cells in patients suffering from Common Variable Immunodeficiency (CVID) [196]. A genome-wide study of host proteins involved in early HIV-1 infection found that siRNA knockdown of BLK could reduce infection of VSV-g pseudoenveloped HIV-1 in 293T cells [289]. However, confirmatory experiments exposing primary B-cells to extracellular HIV-1 proteins such as gp120, Nef or Tat, have yet to ascribe a function for BLK during HIV-1 infection.
1.2.5 Role of c-SRC in HIV-1 Infection

c-SRC is the canonical member of the SFKs, often expressed as a cytosolic proto-oncogene tyrosine kinase. In humans, it is expressed in nearly all cells and tissues, with high levels of protein found in the brain (neuronal splice variant), testis, platelets and PBMCs [190, 290-292]. c-SRC is expressed in CD4+ T-cells 12 hours following TCR-CD3 activation [293]. Its primary functions are thought to play a role in fetal organogenesis, differentiation, cell cycle progression, proliferation, cell survival, cell adhesion, and cell migration via cytoskeletal rearrangement at adhesion networks [294]. c-SRC is a key signaling molecule that integrates multiple signal inputs that induce a variety of downstream signal cascades, as evidenced by the 132 c-SRC mediated phosphorylation sites in the human proteome, 64 known substrates, and over 204 proteins that interact with c-SRC [294]. c-SRC is predominantly repressed in an inactive state, and transiently activated during cellular processes such as mitosis [189]. Conversely, the kinase is constitutively active in abnormal states associated with human cancers and viral infection of chickens by RSV [295, 296]. c-SRC is often associated with cellular membranes, in particular the plasma membrane, endosomal membranes and the nuclear envelope [297, 298]. The defined subcellular locations of c-SRC are important for the regulation of specific cellular events, such as cytoskeletal rearrangement, membrane trafficking and cell cycle progression [189].

Given the essential role of c-SRC in cellular processes, and its potential to cause aberrant tumor growth or differentiation when constitutively active (ex. colon, breast or prostate cancers [296, 299, 300]), it is unsurprising that the cell regulates c-SRC activation with multiple post-translational modifications (see Figure 1.7 for c-SRC protein structure and regulation, originally published in [199]). Phosphorylation of the C-terminal tyrosine residue Tyr530 leads to intermolecular folding and binding to the SH2 domain, suppressing c-SRC tyrosine kinase activity [301]. Interestingly, the regulatory Tyr530 is absent in the truncated v-SRC protein, accounting for its constitutive activity [302]. The conformational change caused by Tyr530 phosphorylation also allows intramolecular binding of the SH3 domain to a proline-rich motif, further suppressing c-SRC activity [303]. A variety of c-SRC binding proteins regulate c-SRC activity by disrupting its SH2 and SH3 intramolecular interactions. For example, PDGFR and
Fig. 1.7: c-SRC domains, tertiary structure, and regulation. (A) The seven domains characteristic of the SFKs are shown for c-SRC. CSK and CHK phosphorylation of Tyr530 can lock c-SRC in an inactive state [199]. (B) Crystal structure of activated c-SRC. The protein domains are the same colours as in (A) [199]. (C) Schematic of activated myristilated c-SRC at the plasma membrane. (D) Inactive state of c-SRC.
Focal Adhesion Kinase (FAK) can bind the c-SRC SH2 domain and activate the enzyme [183, 304], while Sin is a protein that can bind the SH3 domain to activate c-SRC [305]. Moreover, dephosphorylation of Tyr530 by cellular Protein Tyrosine Phosphatases (PTPs) relieves this intramolecular inhibition of c-SRC kinase activity [306]. Full activation of the enzyme requires a second major tyrosine residue, Tyr419, located in an activation loop in the catalytic domain [302]. Phosphorylation of c-SRC at Tyr 419 displaces this residue from the substrate binding pocket, permitting the kinase to phosphorylate substrate targets. Tyr419 can be phosphorylated by other tyrosine kinases or through autophosphorylation [307]. Therefore, phosphorylation of Tyr419 acts as a positive regulator of c-SRC activity, while phosphorylation of Tyr530 acts as a negative regulator to suppress c-SRC activity [302].

c-SRC has multiple phosphatase and kinase binding partners that tightly control its kinase activity. Phosphatases that dephosphorylate c-SRC Tyr530 to activate the enzyme include: Receptor-type tyrosine-protein phosphatase alpha (PTPRA, PTP-α) and PTP non-receptor type 6 (PTPN6 or SHP-1), 11 (PTPN11 or SHP-2) and 12 (PTPN12 or PTP-PEST) [306, 308-310]. C-terminal SRC Kinase (CSK) and C-terminal SRC kinase-Homologous Kinase (CHK) are two kinases that counteract these effects at Tyr530 [191, 311]. In resting T-cells, CSK binding protein (Cbp) and phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) are constitutively phosphorylated, recruiting CSK which prevents the activation of c-SRC [312]. In contrast, T-cell activation induces rapid dephosphorylation of Cbp/PAG, reducing PAG-mediated inhibition of c-SRC by CSK [312].

As previously mentioned, the subcellular location of c-SRC has important function in the signaling cascades that it regulates. Myristoylated c-SRC associated with the plasma membrane regulates cell growth and cell proliferation downstream of growth factor receptors [304]. Plasma membrane c-SRC also localizes at focal adhesion plaques and adheren junctions between cells [313], which is dependent on an intact actin cytoskeleton [314]. c-SRC is also localized to the perinuclear Golgi region of the cell, accounting for 30-40% of the total c-SRC cellular protein [315]. Within the nucleus, c-SRC has been proposed to regulate cell cycle progression and entry into mitosis by interacting with proteins that regulate cell division [298]. It has been shown that the SH3 domain of c-SRC interacts with Sam68, a nuclear protein with 6 proline-rich regions
and a tyrosine-rich C-terminal domain [316]. Sam68 is a multifunctional RNA-binding protein that is required for transactivation of Rev function and the cytoplasmic translation of HIV-1 RNA into proteins [317]. Cytoplasmic mutants of Sam68 have been shown to selectively inhibit the translation of HIV-1 RNA transcripts [317]. This suggests Sam68 directly participates in the nuclear exportation of unspliced and singly-spliced HIV-1 RNA. Moreover, overexpression of Sam68 can enhance the expression of viral p24, through translational regulation of HIV-1 RNA [317]. Beyond interactions with Sam68, c-SRC has also been found within the nucleolus [298]. Despite all this localization knowledge, little is known of the cytosolic effects of c-SRC signal transduction away from the plasma membrane, or the specific substrates and binding proteins that interact with cytosolic c-SRC. Moreover, the potential role of cytosolic c-SRC during the lifecycle of HIV-1 infection has not been established or well defined.

Previous work has demonstrated that the Jurkat T-cell line exhibits robust c-SRC activity after 30 minutes of HIV-1\textsubscript{IIIB} infection [214]. Moreover, treatment of activated PBMCs from healthy human donors with a synthetic peptide expressing the CD4-binding region of gp120 (Peptide T), could induce an 11-fold increase in c-SRC kinase activity within 15 minutes [318]. As with other SFK members, viral Nef binds to c-SRC through allosteric displacement of SH3 domain interactions, requiring the PXXPXR motif on c-SRC [164]. Yet the direct effect of Nef on c-SRC activity \textit{in vivo} during infection is not clear [164, 319]. Relative to HCK, c-SRC lacks the SH3 domain Ile residue that promotes high Nef binding affinity [164]. This creates an SH3 domain more constrained by hydrogen bonds, and may take a conformation less compatible for Nef interactions [164]. In this study, Nef binding to c-SRC alone could not induce strong c-SRC kinase activity \textit{in vitro} [164]. Yet in another paper on immortalized podocytes, Nef was shown to increase c-SRC activity and induce proliferation in this cell line by inducing activation of STAT3, the Ras family of small GTPases (Ras) and MAPK1[320]. These Nef-c-SRC interactions underpinning aberrant glomerular podocyte growth may contribute to HIV-1 associated nephropathy, which can lead to chronic renal failure in patients living with HIV-1 [320].

Kinase inhibitor experiments have also suggested a potential role for c-SRC during HIV-1 infection. A study examining the transfer of HIV-1 from primary DCs to CD4\textsuperscript{+} T-cells
demonstrated that CD4+ T-cells pre-treated with the broad SFK inhibitor PP2, reduced the p24 produced after 6 days of HIV-1JR-CSF or NL4-3balenv infection [264]. However, it is difficult to ascertain the direct effects of c-SRC inhibition with pyrazolopyrimidines, such as PP1 or PP2, as they inhibit LCK and FYN at similar IC50 values as c-SRC [184]. PP1 and PP2 also have many off targets, such as CSK, which can counteract the effect of either drug on SFK activity in cells [184]. Research into the effects of LCK, FYN and c-SRC in the budding of HIV-1 virus-like particles suggests that LCK and FYN both facilitate this step of the viral lifecycle, where as c-SRC had no effect on virus released from transfected 293T cells [227]. Thus c-SRC likely affects HIV-1 replication at an earlier stage of the viral lifecycle. Another study investigating the permeability of endothelial cells in the dissemination of HIV-1 found that pre-treating Lung Lymphatic Endothelial Cells (L-LECs) with SRC inhibitor 1 (SRC-I1) induced hyperpermeability of the lymphatic endothelial barrier [321]. Moreover HIV-1 gp120 could induce similar L-LEC hyperpermeability that required SFK modulation of the cytoskeleton [321]. However, the direct effects of c-SRC during the early entry events of HIV-1 in CD4+ T-cells, and whether they modify the host cytoskeleton, has not yet been determined.

c-SRC involvement in cytoskeletal organization and membrane trafficking via its binding partner, protein tyrosine kinase 2 beta (PTK2B), is a potential signaling pathway exploited by HIV-1 during early infection [147, 197, 322]. PTK2B/c-SRC activity has also been implicated in the migration and podosome formation of immature DCs exposed to soluble gp120, which may enhance the mucosal transmission of HIV-1 as these cells migrate in response to HIV-1 envelope protein [323]. Immature DCs treated with gp120 enhanced the phosphorylation of c-SRC and PTK2B, which activated Rac-1 subfamily of Rho small GTPases (Rac-1) signaling and subsequent paxillin phosphorylation, an essential adhesion molecule that facilitates podosome ring structure [323]. Whether PTK2B/c-SRC signalling plays a similar role in the migration of CD4+ T-cells during HIV-1 infection, has yet to be evaluated.
1.2.6 Role of PTK2B in HIV-1 Infection

PTK2B, the second member of the FAK family, is a 112 kDa non-receptor tyrosine kinase highly expressed in hematopoietic, neuronal and epithelial cell lineages [324-326]. Slightly smaller than FAK, the two proteins share 45% sequence identity [327] (see Figure 1.8 for PTK2B structure, originally published in [328] and [329]). An alternatively spliced form of PTK2B is expressed in the spleen, with a 42 amino acid deletion within the C-terminal domain [324]. PTK2B contains tyrosine phosphorylation sites that recruit binding of SH2 domains of proteins such as Growth Factor Receptor-Bound protein 2 (GRB2) and c-SRC [197]. In addition, the C-terminal end of PTK2B contains two conserved proline-rich motifs that can further bind proteins containing an SH3 domain [330]. The C-terminus also contains a Focal Adhesion Targeting (FAT) sequence that allows for binding to paxillin or leupaxin [331]. Unique to the FAK family, PTK2B has a central tyrosine kinase domain flanked by an N-terminal Four point 1/Ezrin/Radixin/Moesin (FERM) domain, which regulates PTK2B complex formation and phosphorylation [332]. Like c-SRC, multiple phosphorylation sites tightly regulate PTK2B function. Located in the linker region between the FERM domain and kinase domain is Tyr402, a residue critical to the activation of PTK2B and the recruitment of SFKs [197]. The activation loop in the kinase domain also contains Tyr579 and Tyr580, two amino acids that when phosphorylated, induce maximal PTK2B kinase activity [333].

Various cell models have explored the interaction between PTK2B and c-SRC, which link Gi- and Gq-coupled receptors with downstream effects within the cell [197]. C-terminal dephosphorylation of c-SRC at Tyr530 by PTPs is one of the earliest steps to c-SRC activation [306, 308-310]. The SH2 domain of c-SRC then becomes free to bind ligands such as PTK2B, at the PTK2B autophosphorylation site Tyr402 [197]. The binding of c-SRC to PTK2B regulates both the activation of c-SRC kinase activity and its cellular localization, bringing c-SRC in close proximity of potential substrates. For instance, autophosphorylation of PTK2B creates a binding site for the c-SRC SH2 domain, localizing c-SRC to focal adhesions where it can phosphorylate paxillin to regulate cell migration [183].
Fig. 1.8: PTK2B domains, tertiary structure, and regulation. (A) PTK2B protein domains. The c-terminus FERM domain negatively regulates catalytic activity. The FERM domain is followed by a proline-rich region (PRR), and Tyr402 that when phosphorylated, can bind the SH2 domain of c-SRC. Next is the kinase domain with two positive regulatory tyrosines (Tyr579 and Tyr580), and two more PRRs. The N-terminus ends with a focal adhesion targeting (FAT) domain that can bind paxillin at focal adhesions [328]. (B) Crystal structure of the PTK2B kinase (cyan) and FERM (green) domains. The three lobes (F1-F3) of the FERM domain are shown, as well as Ca^{2+}/calmodulin binding motifs (orange) [329]. (C) Schematic of activated PTK2B downstream of voltage-gated Ca^{2+} ion channels.
PTK2B is most well studied for its participation in integrin receptor signaling pathways at focal adhesions in adherent cell types [334], signaling during apoptosis [335], cell migration [326], as well as responding to elevated calcium signals [336]. Extracellular matrix proteins binding to integrins are important for transducing signals that promote cell growth, cell survival, and cell migration. In this integrin pathway, PTK2B colocalizes with integrin receptors at cell contact sites, termed focal adhesions, to phosphorylate structural proteins such as paxillin, which directly associate with the actin cytoskeleton [334]. PTK2B also recruits SFKs and other focal adhesion proteins to activate signaling targets such as Rac1, a member of the Rho Guanosine Triphosphatase (GTPase) family [337]. In the absence of integrin signaling that promotes adhesion-dependent cell survival, PTK2B activation can induce early apoptotic signaling by activating caspase-3, SFKs, and increase DNA fragmentation [335]. In conjunction with integrin receptors, PTK2B transduces migratory signals downstream of growth factor, antigen, chemokine and cytokine receptors [147, 326, 338]. PTK2B is increasingly being recognized for its role in responding to elevated intracellular calcium signals as a Calcium-Dependent Tyrosine Kinase (CADTK). An influx of extracellular calcium via voltage-gated calcium channels or cytosolic release from intracellular stores can induce an increase in PTK2B tyrosine kinase activity [339]. It has also been suggested that calpains, Ca\(^{2+}\)-dependent cytosolic cysteine proteases, cleave PTK2B in order to regulate cell attachment and motility through assembly and disassembly at focal adhesions [336].

Within T-cells, PTK2B has many important roles in T-cell stimulation and effector cell functions [340, 341]. For instance, PTK2B is essential for LFA-1 dependent CD8\(^+\) T-cell activation, migration and cell adhesion [341], forming micro-adhesion rings around the TCR to stabilize the immunological synapse [342], establishing cell polarity [180], and downregulating activated surface receptors. In helper CD4\(^+\) and cytotoxic CD8\(^+\) T-cells, TCR and integrin stimulation lead to tyrosine phosphorylation of PTK2B, enhancing its catalytic activity, and causing PTK2B translocation to sites of cell contact [343]. PTK2B also participates in downstream signaling of TCR and CD28 costimulation that promote IL-2 production [344]. Unique in T-cells, PTK2B exhibits a biphasic burst in activity shortly after TCR engagement that is dependent on SFK activation [340]. Both Tyr402 and Tyr580 on PTK2B are phosphorylated early (5 min) and then...
late (30-60 min) after TCR activation, causing two distinct phases of paxillin phosphorylation and actin polymerization [340]. It has been hypothesized that the two periods of PTK2B activation control the cytoskeletal assembly and then disassembly necessary for optimal T-cell/APC contact, which occurs for approximately 1 hr [340]. In the first burst, PTK2B facilitates cytoskeletal changes that may reinforce the T-cell and APC contact, while the second burst of PTK2B activity disassembles the actin cytoskeleton, and may help disengage the T-cell from the APC.

Past research suggests PTK2B signaling may influence HIV-1 binding and entry of CD4+ T-cells through signaling downstream of G protein-coupled receptors engaged during viral co-receptor binding [147], and responding to ATP that enhances viral membrane fusion to the target T-cell [345]. After natural stimulation of surface CCR5 with Chemokine (C-C motif) Ligand 5 (CCL5) or MIP-1β, or CXCR4 stimulation with SDF-1α, PTK2B becomes rapidly phosphorylated [147]. This suggests that HIV-1 binding to either surface receptor might mimic natural ligand engagement and produce physiologically similar signals. Indeed, infecting the T-cell line DU6 (CCR5+) with JR-FL (R5) pseudoenveloped virus induced PTK2B activation, as did mixing HL60 cells (CXCR4+) with 293T cells expressing HXB2 (X4) envelope protein [147]. Yet whether PTK2B activation is simply a byproduct of HIV-1 envelope engagement, or has a functional role in the intracellular lifecycle of HIV-1 post-fusion, has yet to be determined.

Binding of HIV-1 gp120 to CD4 and a chemokine receptor also induces mechanical membrane stress, stimulating the release of ATP through pannexin-1 hemichannels [345]. In the extracellular space, ATP then acts as an autocrine and paracrine signal, activating the purinergic receptor P2Y2 on the cell surface, which are coupled to G-proteins that recruit PTK2B that subsequently autophosphorylates at Tyr402 to become activated [345]. P2Y2 also has an SH3 binding domain, which can directly recruit PTK2B and induce its phosphorylation and c-SRC activation [182]. These provide even more avenues by which PTK2B recruits signaling complexes that contain SFKs or actin interacting proteins that may enhance HIV-1 fusion upon entry.

Once HIV-1 enters a T-cell, studies suggest FAK apoptotic signaling is blocked [346], and that PTK2B promotes cell migration during HIV-1 infection [323]. After gp120 engages CD4 and
CCR5, FAK localizes to the inner face of the plasma membrane at these receptors [346]. It then becomes phosphorylated and cleaved by caspase-3 and caspase-6, promoting apoptosis and inactivating FAK in uninected cells [347, 348]. Interestingly, the interaction of the FAT domain of FAK with the cytosolic portion of CD4 shares similarity with the interaction of HIV-1 Nef with CD4 [346]. Both FAK and Nef bind CD4 in the same region and with similar affinity [349], yet cause opposing signalling effects. The FAK-CD4 interaction with gp120 can trigger apoptosis of a bystander T-cell, through FAK cleavage by caspases [348]. However, the Nef-CD4 interaction can block this pro-apoptotic signal, permitting an infected T-cell to survive and productively produce virions [346]. Whether PTK2B has a similar role as FAK, to induce apoptosis signaling that is blocked by Nef in CD4+ T-cells during HIV-1 infection, has yet to be assessed. In addition, the potential effect of PTK2B activation on CD4+ T-cell migration during HIV-1 infection has not been determined. As mentioned earlier, gp120-induced transendothelial migration of immature DCs relies on c-SRC signalling, as well as downstream signaling by PTK2B, paxillin, and Rac1 [323]. In these cells, PTK2B activates the Rho GTPase Activated CDC42 Kinase 1 (ACK1), promoting actin polymerization, ring formation, and nucleation of actin filaments within the podosome core [323]. Taken together, there are multiple mechanisms by which c-SRC and PTK2B signaling could be cooperating (or acting independently) to alter intracellular HIV-1 infection post-entry in CD4+ T-cells.
1.3 Ebola Virus (EBOV)

1.3.1 ZEBOV Epidemiology, Transmission and Replication Cycle

In 1976, WHO medical staff and USA Center of Disease Control (CDC) researchers witnessed two separate outbreaks of an unknown contagious disease in Nzara, southern Sudan (284 cases, 151 deaths), and in Yambuku, northern Zaire (318 cases, 280 deaths) [350, 351]. The disease caused fever, joint and muscle pain, rash, abdominal pain, diarrhea with blood, and rapid progression to death, leading to regional panic in both locations. A new virus was isolated by Dr. Peter Piot in Belgium and further characterized by a CDC researcher, Dr. Karl Johnson, from the blood sample of an infected Belgium nun in Zaire [352]. The virus was named after the Ebola River located near the original Zaire outbreak, in what is now the Democratic Republic of the Congo [352]. It was later discovered that both outbreaks were caused by two distinct species of ebolavirus, now classified as Sudan ebolavirus (SUDV) and Zaire ebolavirus (ZEBOV) [353].

Three more have been isolated in the Ebolavirus genus: Bundibugyo ebolavirus (BDBV), discovered in Uganda [354], Reston ebolavirus (RESTV) from the Philippines that infects cynomolgus macaques and is non-pathogenic to humans [355], and Taï Forest ebolavirus (TAFV), isolated from a chimpanzee in Côte d’Ivoire [356]. From 1976 to 2013, there have been 24 separate Ebola outbreaks primarily clustered in central Africa, with mortality rates ranging between 25-90% [6, 357]. The difference in host fatality and disease severity attributed to each ebolavirus strain is still under investigation [357]. None of these outbreaks compare with the unprecedented 2014-16 ZEBOV epidemic in West Africa that led to 28,616 cases and 11,310 deaths in Liberia, Guinea and Sierra Leone (see Figure 1.9, originally published in [5]). This spurred significant developments in Ebola virus therapeutics and vaccines that were tested for safety and efficacy in human trials during the outbreak. While a licensed treatment has proven elusive [25], a successful vaccine candidate has recently been announced [26]. Significant challenges were faced with implementing clinical trials in resource limited settings, including global support and preparedness, maintaining public trust, ethical concerns of implementing randomized control trials during an outbreak, and a decline in Ebola cases once clinical trials were initiated [25].
Fig. 1.9: Spread of ZEBOV in West Africa during 2014-16. The index case was a small boy in Guinea from the Guéckédou prefecture, who died in December 2013 (yellow star). EVD then spread quickly from his family to neighboring villages. By May 2014 the virus had spread to Conakry, the capital city of Guinea, and cases were being reported in Monrovia, the capital city of Liberia, by mid June. The outbreak quickly spread to Sierra Leone and intensified in all three countries from July to October in 2014. Smaller outbreaks in Nigeria and Mali were contained. The epidemic began to wane in November 2014 from international control efforts. All known chains of transmission ended by June 2016, a year and a half since the first case in the region [5].
The West Africa ZEBOV outbreak began with cases reported in Guinea during December of 2013, then spread quickly to neighboring countries Liberia and Sierra Leone [358]. From a single introduction into the human population, it caused an overall fatality rate of 40% over several months [5]. The variant responsible for the epidemic was isolated and determined to be genetically related to ZEBOV, classified as the EBOV-Makona reference strain [359]. In August 2014 an unrelated outbreak of ZEBOV, caused by the variant EBOV-Lomela, occurred in the Democratic Republic of the Congo [359]. Transmission of this variant lead to a shorter ZEBOV epidemic, with fewer fatalities (66 cases, 49 deaths) [359]. It was not until March 29, 2016 that the WHO declared an end to the Public Health Emergency of International Concern regarding the ZEBOV outbreak in West Africa, although sporadic cases continue to occur in Guinea and Sierra Leone [5, 360]. It is not yet understood why some Ebola survivors are asymptomatic seroconverters, while others succumb to life threatening conditions collectively called Ebola Virus Disease (EVD) [361]. Studies of large patient cohorts demonstrate that overall survival and disease severity could be predicted from initial viral load at time of admission to an Ebola Treatment Center (ETC) [18, 19]. The unprecedented outbreak also produced the largest group of Ebola survivors, with ~ 17,000 recovered patients in the three most affected countries, who exhibit newly described chronic afflictions called post-Ebola disease syndrome [362]. Ebola survivors are at greater risk for ocular problems (blurred vision, retro-orbital pain), loss of hearing, difficulty swallowing, abdominal and back pain, fatigue, severe headaches, memory problems, and confusion, among others [362-364]. After acute infection, ZEBOV has been found to persist in semen, ocular fluid, cerebrospinal fluid, placenta, and amniotic fluid [365-367]. A case of a woman contracting ZEBOV in Guinea after sexual intercourse with a male Ebola survivor 470 days after the onset of his symptoms, who also shed ZEBOV in his semen up to 531 days after disease onset, demonstrates that infectious virus can be transmitted sexually from Ebola survivors [360].

Ebola viruses are in the family Filoviridae, and are lipid-enveloped, heavily glycosylated, filamentous RNA viruses (Figure 1.10, originally published in [368]). The 19-kb genome consists of a non-segmented, negative-sense single strand of RNA ((-)-ssRNA) coding for 7 genes, each separated by short intergenic regions [369]. Flanking both ends of the genome
**Fig. 1.10: ZEBOV virion and genome structure.** (A) The negative sense, single-stranded RNA genome of ZEBOV is bound to nucleoprotein (NP) minor nuclear protein (VP30), RNA-dependent RNA polymerase L (L) and polymerase cofactor protein (VP35). Trimeric glycoprotein (GP1,2) pass through the lipid envelope and facilitate viral entry into cells. Matrix proteins VP40 and VP24 are located on the cytosolic side of the lipid envelope. Filamentous ZEBOV virions have variable length, and multiple nucleocapsids are packaged within a single virus particle. (B) Schematic representation of the 7 gene coding regions of the ZEBOV genome, flanked by leader and trailer sequences. Variants of GP protein are indicated [368].
are extragenic regions called the leader and trailer sequences, which signal for encapsidation and contain promoters for replication and transcription [370]. The genome encodes for 4 structural proteins (nucleocapsid protein (NP), glycoprotein (GP\textsubscript{1,2}), viral protein 24 (VP24) and viral protein 40 (VP40)), and 3 replication proteins (RNA-dependent RNA polymerase L (RdRP L), viral protein 35 (VP35) and viral protein 30 (VP30)) [369]. GP\textsubscript{1,2} is the only surface glycoprotein that mediates virus fusion and entry, and the mature trimer of GP\textsubscript{1}-GP\textsubscript{2} heterodimers shares structural similarity with retroviruses such as HIV-1 [371]. Transcriptional stuttering of the polymerase also produces two soluble GP variants called sGP and small sGP.

In the EBOV cellular replication cycle, a variety of host surface receptors act as cofactors prior to viral entry (see Figure 1.11, originally published in [368]). These include T-cell Immunoglobulin and Mucin Domain 1 (TIM-1), integrins, asialoglycoprotein expressed on hepatocytes, and C-type lectins such as DC-SIGN on dendritic cells and macrophages [372-374]. Upon GP\textsubscript{1,2} binding a surface cofactor, the virus enters the early endosome by macropinocytosis [375]. The endosome vesicle acidifies, and host cysteine cathepsins cleave GP\textsubscript{1}, which unmask GP\textsubscript{2} [376]. This allows host Niemann-Pick C1 (NPC1) to bind the cleaved GP (GP\textsubscript{CL}) as the bonafide entry receptor, triggering a conformational change in GP\textsubscript{2} that promotes fusion of the late endosome and viral membranes, releasing the virus into the cytoplasm [376, 377]. Within 6-12 hours, the RdRP L forms a complex with NP in the form of cytoplasmic inclusion bodies outside the nucleus [378]. Polymerase L performs primary transcription of individual mRNAs from the negative-sense RNA genome, with assistance of VP30 (transcription activator) and VP35 (polymerase cofactor), and the mRNAs are capped at the 5’ end and polyadenylated at the 3’ tail [379, 380]. Each gene is transcribed sequentially from 3’ to 5’, producing abundant transcripts of genes at the 3’ end relative to the 5’ end [370]. Thus NP transcripts are the most abundant, while RdRP L transcripts are the least transcribed. Phosphorylation of VP35 triggers RdRP L, NP and VP30 to switch to genomic replication, producing full-length positive-sense, anti-genomic RNA that serve as templates for negative-sense genomic synthesis (secondary transcription) [381]. Newly translated NP, VP35, VP30 and RdRP L proteins associate with the progeny negative-sense RNA strands, while GP and sGP mature separately in the endoplasmic reticulum and Golgi bodies to be
Fig. 1.11: The intracellular ZEBOV replication cycle. ZEBOV GP$_{1,2}$ binds to glycoproteins such as TIM-1 on the host plasma membrane, triggering macropinocytosis of the virus. Host cathepsin B and L cleave GP1, which exposes the entry receptor on GP2 that binds host NPC1. This interaction induces fusion of viral and vesicular membranes in the late endosome. In the cytosol, viral polymerase L performs primary transcription to produce mRNAs, which are translated into proteins that collectively form a viral factory (inclusion body) outside the nucleus. VP35 and VP24 also dampen interferon signaling and RNA-sensing mechanisms, allowing abundant viral proteins and genomic (-)ssRNA to accumulate at the plasma membrane through host Endosomal Sorting Complexes Required for Transport (ESCRT) proteins. Nucleocapsid protein (NP) encapsidating ZEBOV genomic RNA, viral matrix protein VP24, and matrix protein VP40, together mediate assembly and budding of mature virions from the cell [368].
post-transcriptionally modified [378]. Concurrently, membrane-associated matrix proteins VP24 and VP40 are translated and localize to the plasma membrane [382]. When sufficient levels of NP and negative-sense RNA genomes are produced, VP24 helps assemble viruses at the plasma membrane, then VP40 induces budding of the newly formed virions [382]. Outside of replication, VP24 and VP35 have additional roles that help EBOV evade host innate immune responses, chiefly by antagonizing type I IFN pathways [383].

ZEBOV is transmitted between humans by direct contact with mucosal membranes or bodily fluids (saliva, sweat, vomit, diarrhea, blood or semen) of an infected or deceased individual [367]. Following a 6-12 day incubation period, a symptomatic patient will be highly contagious during the acute phase of EVD. Early symptoms consist of fever, chills, malaise and muscle pain, which are non-specific and can be confused with other hemorrhagic fevers or viral infections [357, 367]. When the virus enters broken skin or mucosal surface, antigen-presenting cells are the preferred initial cellular targets, such as monocytes, macrophages or dendritic cells [384]. In vitro infection of these cells demonstrates robust expression of inflammatory mediators IL-1β, IL-6, IL-8, MIP-1α, MIP-1β, MCP-1, and TNF-α [384-386]. Infected cells also release chemokines that recruit additional monocytes and macrophages to the site of infection, which in turn become infected [384]. Symptoms at this stage are gastrointestinal (vomiting, diarrhea, abdominal pain, nausea, anorexia), respiratory (nasal discharge, cough, shortness of breath, chest pain) and vascular (postural hypotension, edema), demonstrating multisystem involvement [387]. As the host immune system attempts to mobilize an adaptive response, ZEBOV may be impairing the essential role of APCs to stimulate T-cell and B-cell responses required to clear infection [388]. Moreover, infected APCs secrete soluble GP, which may act as decoys that counteract neutralizing antibodies [389]. Infection of APCs, coupled with elevated proinflammatory cytokines and a weak adaptive immune response, are likely why ZEBOV can disseminate systemically to infect hepatocytes, adrenal cortical cells, and endothelial cells [390]. This results in hepatocellular necrosis that decreases secretion of coagulation proteins, and abnormal adrenal cortical cells that can no longer maintain blood pressure homeostasis.

In the late stages of illness, symptoms include intravascular volume depletion, hypoperfusion, electrolyte perturbations and renal impairment [387]. Severe lymphopenia and lymphoid tissue
destruction occur, with apoptosis causing the greatest loss of peripheral blood CD4$^+$ and CD8$^+$ T-cells [391]. In concert, the large release of TNF-α from infected monocytes and macrophages induces endothelial permeability, causing vascular leakage and hemorrhage [384]. Together the blood leakage, extensive cytokine release and viral replication in endothelial cells lead to hemorrhage syndrome, often characterized by gastrointestinal bleeding [387]. At the terminal stage of disease, multi-organ failure and shock are the main causes of death.
1.3.2 ZEBOV Clinical Trials, Potential Vaccines and New Therapeutics

Despite international efforts to accelerate research into ZEBOV therapeutics and conduct clinical trials during the 2014-16 West Africa outbreak, there remains no licensed treatment for EVD [25]. However, there was success with a clinical trial testing a preventative vaccine [26]. A variety of vaccine platforms have been used to treat EBOV infection in Non-Human Primate (NHP) models of infection, including: virus-like particle antigen-based vaccines, replication-deficient DNA and recombinant Adenoviral Vector (rAD) vaccines, and replication-competent viral vectors that employ recombinant parainfluenza, rabies, Cytomegalovirus (CMV) or Vesicular Stomatitis Virus (VSV) [392]. However the two most promising vaccine candidates that reached phase II/III clinical testing during the West Africa ZEBOV outbreak were ChAd3-EBO-Z and rVSV-ZEBOV-GP [26].

ChAd3-EBO-Z is a non-replicating, recombinant, chimpanzee adenovirus type-3 vector-based vaccine expressing ZEBOV GP [393]. Earlier studies on cynomolgus macaques demonstrated that single immunization 5 weeks prior to ZEBOV challenge led to complete protection, but this dropped to 50% protection when the animals were challenged 10 weeks post-immunization [32]. Pre-existing immunity to ADVs, which also occurs in humans, likely explains the lower than desired GP-specific humoral response. Thus a booster vaccine with modified vaccinia Ankara expressing ZEBOV GP (MVA-BN-Filo) was administered 8 weeks after ChAd3-EBO-Z immunization, leading to complete ZEBOV protection in macaques challenged as late as 10 months post-immunization [32]. In September 2014, the US National Institute of Allergy and Infectious Diseases (NIAID) and UK Welcome Trust promptly tested the safety, tolerability, and immunogenicity of ChAd3-EBO-Z in 91 healthy participants in two, single-blind, dose-escalation phase I trials in Mali and the USA [393]. Another phase I trial was performed on 60 patients in the United Kingdom, and it was found that the vaccine boosted with MVA elicited strong B-cell and T-cell responses, with no safety concerns reported [394]. The vaccine platform then entered a phase II/III trial called Partnership for Research on Ebola Vaccines in Liberia (PREVAIL), which enrolled 27,000 healthy adult participants, and is estimated to complete by
June 2020. PREVAIL is a randomized, double-blind, controlled, 3-arm study that is also evaluating the other most promising vaccine platform, rVSV-ZEBOV-GP.

rVSV-ZEBOV-GP is a live attenuated, recombinant, replication-competent vaccine consisting of VSV expressing ZEBOV GP [26]. It has the advantage over non-replicating vaccines by providing longer durability, but possesses the potential risk of reversion to wild type (WT), and could cause complications in people with compromised immunity [357]. No adverse events were observed in 80 NHPs immunized with single dose rVSV-ZEBOV-GP [395], or cause disease in immunocompromised Non-Obese Diabetic (NOD) Severe Combined Immunodeficiency (SCID) mice [396]. The vaccine could protect 100% of macaques from lethal ZEBOV challenge up to 6 months after immunization with a single vaccine dose, and confer 50% protection when administered 30 min post-exposure of ZEBOV challenge [33, 397]. rVSV-ZEBOV-GP safety and immunogenicity in humans was also determined in multiple phase I trials across Europe and Africa in 2014 [398, 399]. Recent findings from a phase III, cluster-randomized ring trial in Guinea and Sierra Leone showed this vaccine to be 100% efficacious in the 7,651 people enrolled [26]. When a case of EVD was confirmed, contacts and contacts of contacts were randomized to either immediate vaccination or delayed vaccination (21 days later). None of the participants that were vaccinated immediately developed EVD within 10 days, compared with 23 contacts in the delayed vaccination clusters [26]. As a potential post-exposure vaccine, rVSV-ZEBOV-GP has been administered twice after ZEBOV needle stick injuries, and neither subject displayed EVD, although they developed fevers [400, 401]. While both ChAd3-EBO-Z and rVSV-ZEBOV-GP are very promising vaccine candidates, individual-level correlates of protection and durability in humans remain to be determined.

In parallel with vaccine research, a variety of experimental treatments and therapeutics were prioritized by the WHO for further evaluation in September 2014, based on in vitro and animal models of ZEBOV infection [6]. These were also considered for emergency phase I or phase II/III trials during the ZEBOV outbreak. In addition to intensive supportive care, treatments included: convalescent plasma [20], antisense siRNA drugs [27], monoclonal antibodies [19], and type I interferon regimens [22]. Without an approved specific therapy, intensive supportive care became the benchmark treatment by providing intravenous fluids, electrolyte solutions, and
oral rehydration to help maintain blood volume [16]. Convalescent plasma, whereby plasma from ZEBOV survivors was transfused twice into patients during acute infection, was administered in a non-randomized comparative study in Guinea [20]. However this treatment required ABO-compatibility and optimal collection time from donors. It was determined after the trial completed that the 84 patients received low levels of neutralizing anti-ZEBOV IgG antibodies from the transfusions [402]. Moreover, convalescent plasma transfusions were not easily amendable to sparsely equipped ETCs, and have yet to demonstrate clinical efficacy [20]. Stable Nucleic Acid Lipid Particles (SNALPS) were developed by Tekmira to deliver siRNA in vivo, to target three ZEBOV mRNAs encoding for RdRP L, VP24 and VP35 proteins [403]. The treatment, TKM-Ebola, could protect 100% of rhesus macaques three days post-exposure to a lethal challenge of EBOV-Makona [21]. However, TKM-Ebola failed to demonstrate efficacy in humans in a phase II, single-arm trial, despite infusions being well tolerated [27]. In addition, siRNA strategies are viral strain and variant-specific, which may not be effective in future outbreaks of EBOV [25]. As another strategy, the Public Health Agency of Canada and Mapp Biopharmaceuticals jointly created a cocktail of three monoclonal antibodies called ZMapp, which was administered to ZEBOV patients in a randomized, controlled phase I trial of 71 patients in Liberia, Guinea, Sierra Leone and the USA [19]. ZMapp plus standard care did not show improved efficacy over standard care alone. The antibody cocktail has also been administered for emergency use in seven ZEBOV patients, of which five have survived [404]. However, the slow production of antibodies from tobacco plants limits the usefulness of ZMapp in future EBOV outbreaks. Recombinant interferon supplementation was also considered for treatment because ZEBOV infection is associated with strong downregulation of type I interferons α and β [405, 406]. IFN- α and IFN- β normally clear infection by activating apoptosis in infected cells and recruiting cytotoxic cells [407]. The single-arm, historically controlled phase II trial testing recombinant interferon β recently completed in Guinea. Nine ZEBOV patients received daily subcutaneous injections of IFN- β-1a, and showed higher survival (67%) when compared with 21 patients who received supportive care alone (19%) [22]. While these results are encouraging, enrollment was limited due to potential risk of interferon treatment exacerbating EVD symptoms [22]. Taken together, these therapeutic approaches demonstrate the logistical hurdles of amending an efficacious and well-tolerated strategy that
works well in small animal and NHP models of EBOV infection, with practical challenges of implementing a clinical trial during an ongoing EBOV outbreak: patient recruitment changes rapidly with local transmission rates, resources can be limited, electricity is often intermittent, and limited personnel who are qualified and trained to safely administer therapies with needles in an ETC. For all of these reasons, oral drugs that directly inhibit ZEBOV replication that are safe, stable in warm climates, affordable, and in abundant supply, were strongly considered for prioritization by the WHO during the ZEBOV outbreak in West Africa [6].
1.3.3 Repositioning Nucleoside Analogue for ZEBOV Inhibition

Nucleoside and nucleotide analogues are one of the most successful classes of antivirals for treating HIV-1, Hepatitis B Virus (HBV), Herpes Simplex Virus (HSV), or Varicella Zoster Virus (VZV) in patients [408-412], and are under clinical evaluation for treating HCV, CMV or influenza virus infections [413, 414]. Pharmacological advantages include: direct inhibition of viral polymerases that transcribe either DNA or RNA, long cellular half-lives due to sequential phosphorylation of nucleoside prodrug to an active nucleotide triphosphate by cellular kinases, and broad tissue distribution [415-417]. While viral and human polymerases typically have selectivity to either Nucleotide Triphosphates (NTPs) or dNTPs, based on the ribose or deoxyribose sugar bound to a nitrogenous base, the ability of synthetic nucleotide analogues to disrupt nascent DNA or RNA chain synthesis is empirically determined for each viral polymerase. Unfortunately the broad-spectrum antiviral ribavirin, active against many RNA viruses that cause hemorrhagic fever, is a weak inhibitor of ZEBOV in vitro. Thus the ZEBOV outbreak in West Africa accelerated the development of many nucleoside/nucleotide analogues for the potential treatment of EVD, namely brincidofovir, favipiravir, BCX4430 and GS-5734 [6, 18, 24, 25, 418, 419].

**Brincidofovir (BCV)**

Brincidofovir, a broad-spectrum antiviral developed by Chimerix, is a covalent lipid conjugate of the nucleotide cidofovir (CDV), a cytosine monophosphate analogue [25, 420]. Cidofovir is an Acyclic Nucleotide Phosphonate (ANP), cautiously administered intravenously to treat CMV infections of the eye in HIV-1 patients [421]. To enhance bioavailability by imitating the digestion of monoacyl phospholipids, CDV has been conjugated to the lipid 3-hexadecyloxy-1-propanol (HDP), making it BCV [422]. This has been found to increase the cellular uptake and tissue distribution of orally administered BCV, with improved safety profile over CDV [423]. BCV associates with phospholipids at the cell membrane where HDP becomes cleaved, releasing CDV into the cytosol [424]. Intracellular CDV then becomes phosphorylated twice by cell enzymes to become CDV diphosphate (CDV-DP), the active form of the drug [425]. CDV-DP competes with the DNA polymerase substrates of CMV and vaccinia viruses, inhibiting DNA
elongation by causing chain termination from its incorporation into the nascent DNA strand [426]. BCV was originally developed to inhibit the replication of double-stranded DNA (dsDNA) viruses, such as CMV and ADV [357]. BCV has broad antiviral activity against many dsDNA viruses, including viruses from the families Herpesviridae, Poxviridae, Polyomaviridae, and Adenoviridae [28]. However, long-term treatment with BCV can lead to escape mutations in the viral DNA polymerases, selecting for BCV-resistant poxvirus or CMV [427]. In 2014, it was discovered that BCV showed activity against ZEBOV replication in vitro, the first example of this drug inhibiting the replication of an RNA virus. An in silico screen identifying drugs that could be repurposed to inhibit ZEBOV replication predicted CDV may inhibit the RdRP L of ZEBOV, suggesting a potential mechanism [428]. Yet an in vitro study on the effects of BCV and BCV-derivatives on ZEBOV replication in cell lines only demonstrated antiviral activity of CDV when conjugated to HDP [28]. This could suggest CDV proper has no ZEBOV antiviral effect, or rather, that higher drug concentrations are needed in comparison with BCV. However these two possibilities have yet to be tested, and the mechanism of inhibition of RdRP L, potentially through RNA chain termination, has yet to be determined for either BCV or CDV.

In vivo animal studies have well documented the antiviral activity of BCV in ADV, poxvirus or Vaccinia Virus (VACV) infections, identifying tolerated doses of BCV that protect against lethal viral challenge and reduce disease symptoms [420, 429, 430]. However, the rapid metabolism of BCV in non-human primates precluded the testing of BCV in gold standard models of ZEBOV infection, complicating the design of human efficacy studies evaluating BCV for treating EVD [25]. Nonetheless in October 2014, the FDA approved BCV for emergency use after ZEBOV exposure. Two of three American EVD patients treated with BCV have recovered after treatment; although their survival cannot be directly linked with BCV due to other interventions conducted during treatment [25, 431]. Both patients were treated with a single 200 mg oral BCV dose, and blood samples were drawn before and after 2 days of treatment [427]. Neither patient showed genetic mutations to the ZEBOV RdRP L sequence, although the viral genome from one patient developed a silent point mutation relative to the EBOV-Makona reference strain [427]. The third EVD patient, a man from Liberia visiting Texas and in the late stages of EVD, died four days after initiating BCV treatment.
Because of advanced clinical trials testing the safety and efficacy of BCV in preventing CMV infection [413, 432], and in vitro data of its antiviral effect against ZEBOV that has not been made public, BCV was prioritized by the WHO in late 2014 as a compound to be evaluated in patients with EVD [24]. The drug became fast-tracked for a single-arm, phase II ZEBOV clinical trial that started in Liberia on January 1st, 2015 [24]. Four ZEBOV patients were enrolled, receiving 200 mg oral BCV on day 0, followed by 100 mg oral BCV on days 3, 7, 10 and 14. Unfortunately all four patients died of illness consistent with EVD, despite no serious or unexpected serious adverse reactions reported from BCV treatment [24]. The single-arm study was terminated on January 31st, 2015 by the manufacturer Chimerix, before BCV efficacy could be determined. This was due to insufficient enrollment and fewer new cases of EVD in Liberia during the trial period [24].

**Favipiravir (FPV)**

Favipiravir (T-705) is another broad-spectrum antiviral, developed by Toyama Chemical Co Ltd., to treat influenza strains that are resistant to conventional antivirals [433]. It is a purine nucleoside analogue currently under investigation in phase III trials of uncomplicated influenza in adults [6]. Within the cell, FPV is ribosylated and phosphorylated by cellular enzymes, becoming the active drug favipiravir-RTP (FPV-RTP) [434]. The active drug inhibits the RdRP of Influenza A Virus (IAV) when two molecules of FPV-RMP are incorporated consecutively during primer extension [434]. FPV-RTP outcompetes both GTP and ATP, preventing further primer extension and terminating RNA chain synthesis [434]. FPV has demonstrated in vitro and in vivo activity against many RNA virus families that infect humans, including negative-sense single-stranded RNA (-)ssRNA viruses (*Orthomyxoviridae, Arenaviridae* and *Bunyaviridae*) and (+)ssRNA viruses (*Caliciviridae, Togaviridae, Picornaviridae* and *Flaviviridae*) [434-440].

Two separate studies have reported activity of FPV against ZEBOV replication in vitro [23, 29]. The first study administered FPV to Vero E6 cells (African green monkey kidney epithelial) 1 hour prior to infection with ZEBOV-Mayinga, at a Multiplicity of Infection (MOI) of 0.01 [23]. Five days thereafter, infectious particles were quantified by an immunofocus assay using anti-
EBOV polyclonal antibodies, and cell viability measured by the MTT assay. In cell culture, FPV was able to suppress ZEBOV infection with an IC\textsubscript{50} of 67 μM and an IC\textsubscript{90} of 110 μM, with no affects on cell viability [23]. In the second report, Vero C1008 cells were infected with ZEBOV-E718 or ZEBOV-Kikwit at a higher MOI of 0.1, and then treated immediately with FPV [29]. Infection was visualized by plaque layer formation, and FPV could inhibit ZEBOV plaque formation with an EC\textsubscript{50} of ~200 μM and an EC\textsubscript{90} of ~400 μM [29], higher doses than the previous report, which assessed FPV pretreatment and a lower ZEBOV MOI [23].

To plan for potential human studies, both reports then examined the efficacy and pharmacokinetics FPV in small animal models of ZEBOV infection [23, 29]. This was followed by a third study investigating the pharmacokinetics of FPV in non-human primates [441]. In the first publication, 18 IFNα/βR\textsuperscript{−/−} (A 129) knockout mice, which are immunodeficient and susceptible to EVD, were challenged with aerosolized ZEBOV-E718, at a lethal dose equivalent to 1 TCID\textsubscript{50} [29]. All 6 mice given twice-daily (BID) oral FPV at 50 mg/kg starting 1 hr post-exposure, survived for 4 weeks-post challenge, exhibiting transient weight loss but were otherwise normal after 30 days. Conversely, the 12 mice receiving no treatment showed clinical signs of weight loss, severe ruffling, hunched posture and blindness, and all of them died 7-8 days post-ZEBOV exposure [29]. In the second study, which used IFNα/βR\textsuperscript{−/−} (C57BL/6) knockout mice, FPV was administered either 6 or 8 days after ZEBOV exposure [23]. Fifteen mice were infected intranasally with 1,000 Focus-Forming Units (FFU) of ZEBOV-Mayinga, and treated with 150 mg/kg of FPV BID, starting at 6 (N = 5) or 8 (N = 5) days post-infection [23]. Prior to treatment, all mice lost weight rapidly, showed increasing viremia, exhibited elevated symptoms of EVD, and a decrease in body temperature consistent with shock. In the mice starting FPV treatment at 6 days post-infection, all 5 were able to clear infection from blood within 4 days, and all 5 mice recovered during the 3 weeks of post-infection observation [23]. These mice developed anti-EBOV specific antibodies, and CD8\textsuperscript{+} T-cells responding to viral nucleoprotein, suggesting a virus-specific adaptive immune response was mounted in the absence of type I IFN signaling [23]. In contrast, all 5 of the untreated mice died within 10 days of ZEBOV infection [23]. Of the 5 mice starting FPV treatment 8 days-post infection, treatment delayed death in 1/5 animals, but all of them eventually died of infection by day 15. This
provides evidence of a critical treatment window period where once overt symptoms and high viremia are present, FPV may no longer be beneficial at terminal stages of EVD [23]. Lastly, a pharmacokinetics study of daily maintenance doses of FPV (60 to 180 mg/kg BID) in uninfected Chinese or Mauritian cynomolgus macaques, determined that a 20% dose increase would be needed by day 7 to treat EVD, to compensate for higher drug clearance over time in larger animals [441]. This study helped inform dosing regimens that were useful in planning EVD clinical trials testing the efficacy of FPV.

With strong anti-ZEBOV effects shown in animal models [23, 29], acceptable safety data from human dose-escalation trials in Japan [442], and large stocks of drug readily available, by September 2014 FPV quickly became short-listed by the WHO for emergency use after ZEBOV exposure. A case study of a 43-year old doctor who contracted ZEBOV in Sierra Leone, and was treated with a combination of FPV and ZMapp in Switzerland, demonstrates the complexity in attributing drug efficacy in non-controlled, emergency treatment settings [443]. While the patient’s viral load decreased rapidly and the patient fully recovered, it is not clear whether immune responses cleared infection, whether either treatment had an effect, or both [443]. To answer such questions, international collaborations in the fall of 2014 led to the rapid planning of the JIKI trial: a multicenter, historically controlled, single-arm phase II clinical trial in Guinea [18]. It recruited EVD patients beginning in December 2014 and completed by April 2015. Patients (N = 126) were offered standardized care and optional oral FPV treatment, with an initial adult loading does of 6,000 mg FPV on the first day, and maintenance doses of 2,400 mg FPV per day for the next 9 days [18]. Of 99 adults and adolescent analyzed, the mortality rate was marginally reduced during the clinical trial (52.6%) compared with the historical mortality rate at the ETC prior to the study (55%), leading to no firm conclusion of the benefits of FPV treatment [18]. FPV was well tolerated by all patients who received it, but the study was unable to determine the efficacy of FPV on EVD mortality or relationship with RNA viral load, suggesting further FPV dose optimization is required [18]. Most importantly, it was observed that baseline viremia was a strong prognostic of patient survival, regardless of treatment: the mortality rate of those with low baseline viremia (< $10^{7.7}$ genome copies/mL) was 20%,
compared with the 91% mortality rate of patients with high baseline viremia (> $10^{7.7}$ genome copies/mL) [18].

After the trial ended, analysis of plasma FPV concentrations from 66 of the treated patients revealed that by day 4 of treatment, the mean plasma concentration dropped and was much lower than anticipated by modeling: 64.4 μg/mL predicted versus 25.9 μg/mL observed [444]. Thus, the JIKI trial did not achieve the target plasma drug exposure level defined before the trial began. Previous pharmacokinetic research in healthy Japanese volunteers shows that single-dose FPV has a short half-life of 2-5.5 hrs [442]. Nevertheless, a retrospective clinical trial case series provides optimism for further FPV efficacy research in treating EVD [445]. In a Sierra Leone hospital, 85 EVD patients were enrolled into supportive therapy (control group), while 39 patients were given oral FPV in addition to supportive therapy [445]. Relative to the control group, patients administered FPV showed greater symptom improvement, reduced viral load, longer average survival time (46.9 days vs. 28.9 days) and a higher overall survival rate (56.4% vs. 35.3%) [445]. These are compelling reasons to design future randomized controlled trials to test the efficacy of FPV in treating EVD, with careful optimization of drug doses to achieve target FPV plasma levels. Currently a phase II, dose-escalation trial (FORCE) is recruiting male Ebola survivors in Guinea, to evaluate whether high doses of FPV can reduce ZEBOV RNA shedding in semen.

**BCX4430**

BCX4430 is an experimental adenosine analogue created by BioCryst Pharmaceuticals, studied for its inhibition of RNA viruses, such as HCV, Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) [6]. Like FPV, BCX4430 can broadly inhibit (-)ssRNA viruses (*Orthomyxoviridae, Arenaviridae, Paramyxoviridae* and *Bunyaviridae*) and (+)ssRNA viruses (*Filoviridae, Togaviridae, Picornaviridae Flaviviridae* and *Coronaviridae*) [418, 446, 447]. It is highly selective for viral RNA polymerases, and has not been shown to incorporate into human RNA or DNA, when human Huh-7 cells are treated with BCX4430 [418].
BCX4430 was originally identified from a small-molecule library of inhibitors designed to target RNA polymerase and reverse transcriptase activity. Inside a cell it is converted to BCX4430-triphosphate (BCX4430-TP), where after pyrophosphate cleavage, becomes incorporated into the nascent viral RNA strand [418]. Its mechanism of action is through non-obligate RNA chain termination, causing premature termination of viral replication or transcription. This has been demonstrated for the HCV RNA polymerase in cell-free experiments, where BCX4430-TP caused premature termination of RNA chain synthesis two bases after BCX3330-MP incorporation into the growing RNA chain [418]. Within various cell lines and primary human hepatocytes, BCX4430 becomes rapidly phosphorylated within hours [418]. In HeLa cells transfected with an EBOV minigenome, which creates transcription and replication competent virus-like particles (trVLPs), viral glycoprotein-GFP expressed on the cell surface was inhibited by BCX4430 administered 5 hrs post-transfection, with an EC$_{50}$ of ~ 12.5 μM. No adverse effects on cell viability were detected by the lactate dehydrogenase (LDH) release assay [418]. In Hela cells infected with EBOV-Kikwit, surface expression of viral GP$_{1,2}$ was completely inhibited by BCX4430 at concentrations greater than 100 μM, with an EC$_{50}$ of ~ 11.8 μM. This was repeated in primary human monocyte-derived macrophages infected with EBOV-Kikwit and treated with BCX4430 (EC$_{50}$ of ~ 32 μM) [418]. Taken together, these in vitro findings suggest BCX4430 inhibits ZEBOV replication; however, they only come from a single report, and have yet to be reproduced independently.

The pharmacokinetics and efficacy of BCX4430 have been tested in both a small animal model and non-human primate models of ZEBOV infection [418, 448]. While the prodrug has been found to rapidly decrease in the plasma of rodents after intramuscular injection (half-life of 5 minutes), the active metabolite BCX4430-TP has an intracellular half-life of 6.2 hours in the liver of rats [418]. In an experiment with immunocompromised C57Bl/6 mice, 30 mice were injected daily with 0.9% saline vehicle (N = 10) or administered 150 mg/kg BCX4430 BID, either by intramuscular injection (N = 10) or orally (N = 10), starting 4 hours prior to infection. The mice were then infected with a mouse-adapted strain of ZEBOV-Mayinga at 1,000 PFU [418]. All mice administered saline vehicle died within 8 days. Meanwhile, intramuscular injections of BCX4430 provided 100% protection against lethal ZEBOV challenge, and all mice
lived for the 14 days of follow-up [418]. Oral administration of BCX4430 was also protective, with 80% of the mice surviving until day 14. In a separate dose-range study in cynomolgus macaques, 3.4 to 16 mg/kg of BCX4430 BID was injected intramuscularly into animals 48 hours post-ZEBOV exposure [448]. Despite the 16 mg/kg dose group showing a significantly prolonged lifespan relative to the control group (13.2 days vs 7.2 days), none of the animals survived [448]. Thus, a second study was performed with higher doses, administered closer to the time of infection. Rhesus macaques were injected with 16 mg/kg BID (N = 6) or 25 mg/kg BID (N = 6) of BCX4430, or injected with saline vehicle (N = 3) after 30-60 minutes of lethal ZEBOV challenge [448]. Following 14 days of follow-up, all animals administered 25 mg/kg BID of BCX4430 survived, while 4/6 animals in the 16 mg/kg BID group survived, and all 3 animals in the control group died by day 9 [448]. The mean peak viral load on day 8 also decreased in both groups of BCX4430-treated animals. Clinical trial data has yet to be reported for BCX4430 in humans, however, a phase I study of the safety, tolerability and pharmacokinetics of daily intramuscular injection of BCX4430 in healthy participants is ongoing [448].

GS-5734

Recently, Gilead has reported that a novel adenosine analogue, GS-5734, inhibits ZEBOV replication with high in-vitro efficacy [6]. GS-5734 is a monophosphoramidate prodrug with broad-spectrum activity against (+)ssRNA viruses (Filoviridae and Coronaviridae), (-)ssRNA viruses (Paramyxoviridae and Arenaviridae), but not against (+)ssRNA Togaviridae or retroviruses such as HIV-1 [417, 449]. It has been shown to have low toxicity in primary human cells and cell lines, with selectivity against viral polymerases [417].

GS-5734 was discovered from a library of nucleoside and nucleoside phosphonate analogues, in a ZEBOV collaboration between the CDC and United States Army Medical Research Institute of Infectious Diseases (USAMRIID) [419]. Focused screening of the ~ 1,000 compounds identified a 2-ethylbutyl l-alaninate phosphoramidate parent drug, which through further optimization by structure activity relationships, led to identifying an isomer that inhibited ZEBOV in human macrophages with an EC\textsubscript{50} = 86 nM, called GS-5734 [419]. Nucleoside
analogue drugs typically have slow kinetics during the first phosphorylation event, whereas GS-5734 was designed to have a monophosphate promoiety, to bypass this rate-limiting step and greatly enhance intracellular triphosphate concentrations of the compound [417]. GS-5734-TP has shown to have a long half-life of 24 hrs in primary human monocyte-derived macrophages, 30-times greater when compared to its parent drug [417]. When GS-5734 was added to HeLa or HFF-1 cells 2 hrs prior to infection with ZEBOV-Makona (5 PFU) or ZEBOV-Kikwit (0.5 PFU), it could markedly inhibit infection measured by immune-staining 2 days later, with low EC₅₀ values: ~0.2 μM for inhibiting ZEBOV-Makona and ~ 0.16 μM for inhibiting ZEBOV-Kikwit [417]. In similar experiments, GS-5734 could inhibit ZEBOV-Makona infection in pretreated primary human macrophages (EC₅₀ = 0.086 μM) and in the hepatocyte cell line Huh-7 (EC₅₀ = 0.07 μM) [417]. At doses greater than 0.1 μM, pretreated Huh-7 cells infected with ZEBOV-Makona for 3 days demonstrated a GS-5734 dose-dependent reduction in viral RNA produced in infected cells. Consistent with the mechanism of action of FPV and BCX4430, GS-5734 causes premature chain termination during RNA synthesis of a viral RdRP, as demonstrated by its cell-free inhibition of the RNA polymerase isolated from Respiratory Syncytial Virus (RSV) [417]. GS-5734 was also found not to inhibit human RNA Pol II or human mitochondrial RNA polymerase, bolstering its selectivity to viral RNA polymerases [417].

In non-human primate models of ZEBOV infection, GS-5734 has exhibited unique properties that make it a strong candidate for further development [417]. Intravenous administration of 10 mg/kg of GS-5734 to rhesus macaques lead to rapid elimination of the prodrug (half-life of 0.39 hours), where as intracellular GS-5734-TP was persistent in PBMCs (half-life of 14 hours) [417]. Tissue distribution of the drug administered to cynomolgus macaques shows that it can reach viral sanctuary sites (testes, epididymis, eyes, and brain) within 4 hours [417], suggesting GS-5734 could be useful for Ebola survivors, who continue to shed virus or suffer from post-Ebola virus syndrome [362]. Efficacy studies of GS-5734 in rhesus macaques found that daily intramuscular injection of 3 mg/kg or 6 mg/kg of drug, starting 30-90 minutes following ZEBOV challenge, were suboptimal in reducing systemic viremia or improving animal survival [417]. However, animals given a 10 mg/kg loading dose of GS-5734 three days-post ZEBOV exposure, followed by daily GS-5734 injections of either 3 mg/kg (N = 6) or 10 mg/kg (N = 6), showed
100% survival after 28 days [417]. All animals administered saline vehicle died by day 9, and the rhesus macaques given 10 mg/kg of GS-5734 daily showed the lowest levels of plasma viral RNA, with no evidence of genetic variants that would indicate selection of GS-5734-resistant virus [417].

Similar to BCV and FPV, GS-5734 is also reserved for emergency use after high-risk exposure to ZEBOV. During the 2014-16 West Africa ZEBOV outbreak, there were two instances of the compassionate use of GS-5734 [450, 451]. The first case was a female nurse from Scotland who contracted ZEBOV in Sierra Leone, and was readmitted to hospital 9 months after suffering acute meningitis and EVD relapse, with detectable virus found in her cerebrospinal fluid [450]. She was treated with corticosteroids and GS-5734 for 14 days, until her viral RNA load became undetectable [450]. The second case was a newborn girl in Guinea, who was diagnosed with EVD the day she was born from a ZEBOV-positive mother diagnosed with EVD [451]. The newborn was promptly administered ZMapp, plasma from an Ebola survivor and GS-5734. After 20 days of treatment, she showed no detectable ZEBOV RNA in her blood, and was discharged by day 33 in good health [451]. This newborn girl is the first documented case of a patient surviving vertical transmission of ZEBOV.

Given the propensity for GS-5734-TP to accumulate in mononuclear cells, the primary target cells of ZEBOV infection [417], this drug may be valuable for post-exposure prophylaxis. The large-scale manufacturing of GS-5734 permitted for phase I clinical trials evaluating the safety and pharmacokinetics of single and multiple doses of GS-5734, administered by intravenous infusion [419]. No serious adverse effects to GS-5734 have yet to be observed. In addition, the distribution of the drug to viral sanctuary sites prompted interest in evaluating GS-5734 for efficacy in treating post-Ebola syndrome, and to potentially reduce persistent viral shedding in the male genital tract [419]. Adult male Ebola survivors are currently being enrolled in a double-blind, randomized, placebo-controlled phase II study (PREVAIL IV), to assess whether 100 mg of daily GS-5734 treatment can cause long-term clearance of ZEBOV and reduce viral shedding in semen [419].
1.3.4 Testing Other Potent Nucleoside/Nucleotide Analogues: Zidovudine, Lamivudine and Tenofovir

The rapid development of preclinical research and EVD clinical trials to test the efficacy of brincidofovir, favipiravir, BCX4430 and GS-5734, demonstrate the escalated interest in nucleoside and nucleotide analogue inhibitors of the ZEBOV RNA-dependent RNA polymerase L. Since the West Africa ZEBOV outbreak began in 2014, several compounds approved for other indications have shown \textit{in vitro} activity against ZEBOV replication [6, 452-454]. In this section, the tertiary structure of RdRP L will be compared with other related viral polymerases, allowing for comparisons to be drawn between nucleoside/nucleotide analogues and NTP binding to RdRP L [455, 456]. This will provide the rationale for considering potent nucleoside analogue inhibitors of HIV-1 and HBV, such as zidovudine (AZT), lamivudine (3TC) and tenofovir (TFV), for testing of antiviral activity against ZEBOV replication. The case for investigating various combinations of nucleoside analogues targeting the RdRP L of ZEBOV will also be made.

**Modeling ZEBOV RdRP L and nucleotide binding**

Given the lack of a defined crystal structure of the EBOV polymerase, two separate modeling studies have used the tertiary structures of related viral RNA polymerases and reverse transcriptases [455], as well as combined homology and \textit{ab initio} modeling [456], to describe putative domains of the RdRP L of ZEBOV. The crystal structures of nucleotide binding sites on RNA and DNA polymerases are of particular interest, as no polymerase has been shown to have absolute template or substrate specificity [457-459]. Moreover, available structures of polymerases, bound either to natural nucleotides or nucleotide analogues, demonstrate similar conserved binding mechanisms [460-462].

In the first study, the ZEBOV protein L sequence together with the crystal structures of 20 RTs from retroviruses and monomeric RdRPs from (+)ssRNA, (-)ssRNA and dsRNA viruses, led to a model of the tertiary protein structure of RdRP L [455]. The general structure of viral RNA polymerases and retroviral reverse transcriptases in Figure 1.12 are from [455]. The crystal
Fig. 1.12: General structure of polymerases from the three main groups of RNA viruses and retroviruses. The conserved fingertips (orange), fingers (yellow), palm (green) and thumb (red) subdomains of four prototypical viral polymerases are shown. (A) Poliovirus RNA-dependent RNA polymerase. (B) Influenza A virus RNA-dependent RNA polymerase. (C) Bacteriophage Φ6 RNA-dependent RNA polymerase. (D) HIV-1 RNA-dependent DNA reverse transcriptase [455].
structures of 20 polymerases from four of the main viral classes share a right-hand appearance, with three characteristic subdomains called the fingers, palm and thumb domains [463]. The thumb domain participates in non-specific interactions with the primer strand, while the palm domain coordinates the phosphoryl transfer reaction of an incoming ribonucleotide to the growing RNA chain [464]. A common mechanism of nucleotidyl transfer is used by RdRP enzymes within the palm subdomain. In this region, a conserved aspartate residue coordinates with two divalent metal ions to catalyze the polymerization reaction of the growing RNA chain [465, 466]. Consequently, this area of the palm subdomain is the most conserved sequence of all viral RdRP. In many cases, a fourth fingertips domain can be present, creating a more closed right-hand structure [455]. This fingertips domain is known to influence the interaction between an incoming nucleotide and the template strand [467].

Specifically within the family of RdRPs, six conserved structural motifs have been identified within the palm subdomain (labeled A-F), containing key residues for proper ribonucleotide binding and protein conformational changes [468-470]. Key residues within the active site have several interactions with an incoming nucleotide. Conserved aspartic acid residues within motifs A and C of the palm subdomain, along with basic residues in motif F of the fingers subdomain, specifically interact with the triphosphate moiety of the incoming nucleotide [468, 471]. An aspartic residue in motif A and an asparagine in motif B of the palm subdomain also interact with the 2'OH moiety of ribonucleotides, providing selection for ribonucleotides in the nucleotide binding pocket of RdRPs [471, 472]. Residues in motifs A and B of the palm and fingers subdomains of RdRPs also interact with the sugar moiety of the nucleotide, playing an additional role in substrate discrimination [468]. Unique to RTs and DNA-dependent DNA polymerases is a bulky side chain that creates a steric barrier preventing ribonucleotides from entering the nucleotide binding pocket [473]. Lastly, the RNA template and primer bases provide many interactions with the incoming nucleotide base moiety in RdRPs, strongly influencing ribonucleotide specificity.

Considered together, the four conserved subdomains of viral RNA and DNA polymerases, and six highly conserved structural motifs within the palm subdomain, allowed for homology-based construction of a general three-dimensional model for ZEBOV L (see Figure 1.13, from[455]).
Fig. 1.13: Predicted structure of the RdRP L of ZEBOV. (A) Alignments of other viral polymerases suggest conserved fingers (yellow), palm (green) and thumb (red) domains are present in polymerase L, with 90% confidence. (B) Close-up of the enzyme active site identifies viral polymerase motifs A-E in the moded of RdRP L [455].
The protein L model was identified to contain the finger, palm and thumb subdomains, with conserved structure in the palm subdomain, consistent with other viral RNA and DNA polymerases [467, 474]. It also showed similar structural motifs with the crystal structures of DNA and RNA polymerases bound to nucleotides or nucleotide analogues, suggesting similar binding mechanisms may be involved [455]. These conserved regions, in particular the palm subdomain which contains the residues for nucleotide binding, could explain why FPV and BCX4430 exhibit broad-spectrum inhibition of many (+)ssRNA and (-)ssRNA viruses, and why BCV was recently found to have activity against RNA viruses [455]. Thus, nucleotide analogues designed to inhibit the active site of polymerases from different viruses, such as 3TC inhibiting HIV-1 RT, may also inhibit ZEBOV RdRP catalytic activity [455].

In the second study, a 3-D model of the middle polymerase domain of ZEBOV protein L (AAs 648-1457) was generated by homology-mediated modelling, based on the known X-ray structures of the viral RNA polymerases of human Enterovirus (EV), Bovine Diarrhea Virus (BVDV) and Foot-and-Mouth Disease Virus (FMDV) [456, 475-477]. This model of the middle domain of protein L shared significant architectural similarities with the polymerases of other RNA viruses, namely Poliovirus, Rotavirus, HCV and Rhinovirus [456]. To determine the putative ribonucleotide binding pocket, the crystal structures of EV and BVDV polymerases complexed with GTP substrate were superimposed on the predicted protein L structure [456] Complementing this approach, the solved structure of FMDV polymerase complex with the nucleotide analogue ribavirin and template-primer RNA, also helped to identify a helical motif at AAs 983-990 as the probable ribonucleotide binding domain: FLRQIVRR [456].

Next, with the predicted structure of this nucleotide binding domain, and conformational information of nucleotides (ATP, CTP, GTP and UTP) and select nucleotide analogues, in silico molecular docking studies of polymerase-ligand complexes and their associated interaction energies could be modeled [456, 478]. The putative nucleotide binding domain appears to have two entry sites, one on the “left” and one on the “right”. The lowest total interaction energy for each natural nucleotide suggests that all four NTPs bind the FLRQIVRR motif from the “left” (ATP = -8.83 kcal/mol, CTP = -11.59 kcal/mol, GTP = -9.23 kcal/mol and UTP = -8.96 kcal/mol) (Figure 1.14, from [456]). Interestingly, triphosphorylated nucleotide analogues were
Fig. 1.14: Model of ribonucleoside triphosphates in complex with RdRP L. Each of the four nucleotides (green) were found to bind the “left” side of the putative nucleotide binding pocket of ZEBOV polymerase L (yellow). (A) ATP. (B) CTP. (C) GTP. (D) UTP [456].
found to be either “left-handed” or “right-handed” in terms of their lowest interaction energy with RdRP L. This separated nucleotide analogues into two distinct docking groups. For instance, abacavir-TP (-10.42 kcal/mol), favipiravir-RTP (-11.51 kcal/mol) and BCX4430-TP (-8.23 kcal/mol) were predicted to bind from the “left”, while tenofovir-DP (-12.51 kcal/mol) is modeled to bind from the “right” (see Figure 1.15 for an example of nucleotide analogues binding differently, from [456]). Moreover, these total energy interaction values of triphosphorylated nucleotide analogues were very similar to the natural NTPs, suggesting they could compete for substrate binding to RdRP L [456]. As an example, the model predicted FPV-RTP would outcompete binding of CTP or UTP to the putative nucleotide binding pocket, while BCX4430-3P would outcompete CTP binding [456]. Potential interactions between AZT, 3TC and TFV with the nucleotide binding pocket were also described [456]. In the following three sections, the established interactions of AZT, 3TC and TFV nucleoside/nucleotide analogues binding and inhibiting viral RdRPs and RTs, their clinical indications for treating other viral infections, and potential for inhibiting the RdRP L of ZEBOV, will be discussed.

Zidovudine (AZT)

In 1985, it was discovered that the thymidine analogue zidovudine, also known as azidothymidine or retrovir, could inhibit HIV-1 replication in vitro [479]. Originally designed to target DNA synthesis in breast cancer, AZT became the first antiviral FDA-approved for the antiretroviral treatment of HIV-1/AIDS in 1986 [480]. AZT shows strong synergy with 3TC in inhibiting HIV-1, and was an important component of first-line cART regimens [481, 482].

As with most other nucleoside analogues, intracellular AZT becomes phosphorylated to its active form (AZT-TP) by host cell enzymes [483]. AZT-TP inhibits HIV-1 reverse transcriptase when it is incorporated into the nascent DNA chain, causing early chain termination [483]. It is a weak inhibitor of cellular DNA polymerases α and β, but can inhibit mitochondrial DNA polymerase γ [484]. This can impair mitochondrial DNA synthesis at high AZT concentrations, causing apoptosis of cardiac and skeletal muscle cells, which can lead to toxic mitochondrial myopathy when used long-term in patients [485, 486]. Research into the mechanism of AZT-TP inhibition
Fig. 1.15: Example nucleoside/nucleotide docking to the ZEBOV RdRP L. (A) Abacavir-TP (red) binds the FLRQIVRR motif (yellow) from the “left” side, similar to favipiravir-RTP and BCX4430-TP. (B) Tenofovir-DP (red) binds the nucleotide binding pocket from the “right” side [456].
of HIV-1 RT revealed that the enzyme undergoes isomerisation in a two-step binding reaction with template-primer, before binding free dNTP substrate [487]. Incorporated AZT-MP into the DNA strand inhibits the ribonuclease H (RNase H) cleavage activity of HIV-1 RT, acting as a competitive substrate inhibitor when RT is in the presence of divalent cation activator Mn\(^{2+}\), or as an uncompetitive substrate inhibitor in the presence of Mg\(^{2+}\) [488]. This suggests the conformation of RNase H is dependent on the divalent cation present, altering its mode of hydrolysis between endonucleolytic and exonucleolytic cleavage [488].

Research into HIV-1 RT resistance mutations towards AZT treatment provides valuable insight into the mechanisms of this drug, suggesting potential means of how AZT might inhibit the RdRP L of ZEBOV. Mutations in the RNase H primer grip region have shown to enhance resistance to AZT, by decreasing template switching, altering the balance between template RNA degradation and nucleotide excision, and changing RT interactions with the template-primer complex [489]. While other RT mutations also permit HIV-1 to overcome AZT monotherapy and increase viral replication, substitutions at amino acids D67N, K70R, T215Y, and K219Q come at the cost of processive DNA synthesis [490]. HIV-1 RT resistant to AZT inhibition also show mutations that enhance the excision of AZT-MP from the end of the primer strand at the nucleotide binding site [491]. These mutations increase AZT-MP excision because of a new binding site that can recruit ATP, which acts as the pyrophosphate donor for AZT-MP-mediated excision [492].

In addition to HIV-1 antiviral activity, AZT has been found to inhibit the replication of a variety of retroviruses, DNA viruses and RNA viruses [493-496]. AZT can inhibit the replication of HBV in HepG2 cells, a virus encoding a DNA-dependent DNA polymerase [493]. The polymerase of HBV can transcribe from RNA as well as DNA templates, and AZT can inhibit in vivo HBV replication in chronically infected patients [494]. Furthermore, in vitro AZT treatment of uninfected PBMCs can prevent infection from Human T-cell Lymphotropic Virus type I (HTLV-1) [495]. The HTLV-1 genome is ssRNA, similar to HIV-1, and encodes a reverse transcriptase enzyme. Human Foamy Virus (HFV), another retrovirus that occasionally infects humans, can also be efficiently inhibited with AZT treatment [496].
With respect to RNA viruses that cause hemorrhagic fever, AZT has also shown strong antiviral activity [497, 498]. Hantaan Virus (HTN) is a (-)ssRNA virus that causes hemorrhagic fever with renal syndrome (HFRS) in humans [499]. The virus is endemic to China and the Korean Peninsula [500, 501]. An *in vitro* study of inhibitors of HTN found that cells treated with AZT 24 hrs post-infection, showed no detectable HTN RNA by qRT-PCR [497]. In addition, a study of an aryl phosphate derivative of AZT, called zidampidine, showed this compound could protect mice from lethal Lassa virus challenge [498]. Lassa virus has a bisegmented, ambisense ssRNA genome [502]. In the zidampidine pilot study, CBA mice were injected with 250 mg/kg of zidampidine prior (-24 hr), during (1 hr) or post-challenge (24, 48, 72 or 96 hr) of intracerebral injections of Lassa virus (Josiah strain) [498]. The zidampidine-treated mice showed significantly higher survival compared to mice administered vehicle [498].

From the model of nucleotide analogue docking to the putative nucleotide binding pocket of ZEBOV RdRP L described in the previous section, it was predicted AZT-TP would have a similar interaction energy to the viral polymerase (-11.47 kcal/mol) as natural NTPs (-8.83 to -11.59 kcal/mol) [456]. Moreover, AZT-TP belongs to the group of nucleotide analogues that would dock at the “right” side of the nucleotide binding motif, opposite of FPV and BCX4430 [456]. AZT-TP was also predicted to outcompete CTP binding directly, providing a potential mechanism of this drug during *in vitro* ZEBOV infection [456].

**Lamivudine (3TC)**

Lamivudine is a nucleoside analogue of cytidine that can inhibit HIV-1 and HBV replication, used for salvage therapy when treating HBV [503-505]. Similar to AZT-TP, the active metabolite 3TC-TP inhibits HIV-1 reverse transcriptase when 3TC-MP becomes incorporated into the growing DNA chain, causing early chain termination [506]. Also similar to AZT, 3TC is a weak inhibitor of human DNA polymerases α and β [506]. 3TC is orally administered, has high bioavailability, and may cross the blood-brain barrier [507]. 3TC is a component of cART regimens to treat HIV-1 in patients, and is highly synergistic in inhibiting HIV-1 when combined with AZT [408, 481].
In 1992, 3TC was first reported to be a potent and highly selective inhibitor of HIV-1 and HIV-2 replication in CD4+ T-cell lines and human PBMCs ex vivo [505]. It was then determined in HeLa cells that 3TC-TP was a direct competitor of dCTP in the RNA-dependent DNA polymerase activity of HIV-1 RT [506]. 3TC-TP can also outcompete dCTP in the presence of human DNA polymerase γ, with 3TC-MP becoming incorporated into mitochondrial DNA [508]. However, this enzyme has substrate exonuclease activity that can remove 3TC-MP from the dsDNA, likely explaining the low levels of mitochondrial toxicity associated with 3TC when compared with AZT [508].

Interestingly, HIV-1 variants that become resistant to 3TC also show reduced processivity similar to AZT-resistant variants; however, it is at a cost to viral replication in infected primary cells [509]. Often these mutations occur at the catalytic core of RT in a conserved YMDD motif, where the methionine residue becomes replaced with isoleucine (M184I), and then valine (M184V) in subsequent HIV-1 quasispecies [509]. Under limiting dNTP concentrations, HIV-1 RT enzymes carrying either mutation produce shorter cDNA transcripts when compared with wild type RT [509]. The decreased processivity of M184I and M184V RT mutants, slower primer extension rate, and increased strand transfer activity can all be attributed to defective dNTP utilization [510]. These alterations are not caused by changes in binding of the DNA primer-RNA template or RNAse H ribonuclease activity. Thus slower cDNA synthesis, coupled with normal dissociation rate of the primer-template, accounts for the lower processivity and shorter HIV-1 cDNA transcripts exhibited in 3TC-resistant HIV-1 strains [510]. HIV-1 RT enzymes carrying the M184I and M184V substitutions also demonstrate increased fidelity, and are more accurate than wild type RT in assays testing misinsertion and mispair extension efficiency [511]. This suggests a trade-off in 3TC-resistant RT enzymes: increased polymerase fidelity comes at the cost of slower cDNA synthesis and reduced synthesis of full-length viral cDNA [511]. Indeed, HIV-1 carrying the RT M184V mutation, which produces more accurately transcribed viral cDNA, is less capable of compensatory mutagenesis that allows reversion back to wild type replication kinetics, impairing viral fitness [512]. The effects of substitutions at Met184 have been further clarified with crystal structures of 3TC-TP bound to M184I mutant HIV-1 RT, and compared with 3TC-TP bound to wild type HIV-1 RT [513]. As a beta-branched
amino acid, the isoleucine mutation creates steric hindrance at position 184, allowing entry of dNTPs to the nucleotide binding pocket but not 3TC-TP [513]. The valine mutation at the same position likely has the same effect, as it is also a beta-branched amino acid. Moreover, the crystal structure of the M184I mutant HIV-1 RT revealed repositioning of the template-primer, suggesting how the 3TC-resistance mutation could be interfering with optimal enzyme polymerase activity [513].

3TC has also shown to effectively inhibit the polymerase activity of the dsDNA virus HBV and retrovirus HTLV-1, similar to AZT [514, 515]. In experiments with duck HBV, 3TC-TP was shown to cause DNA chain termination as a competitive inhibitor of the viral DNA polymerase, with respect to dCTP [514]. However, development of HBV resistance mutations such as M552V, M552I or L528M, precludes 3TC as a first-line therapy for HBV infection in patients [516]. It appears that mutations to HBV DNA polymerase do not confer 3TC resistance through removal of 3TC-MP by an exonuclease mechanism [517]. These mutations likely cause steric hindrance with their side-chains, preventing 3TC-TP access to the nucleotide binding pocket, similar to HIV-1 RT resistance to 3TC [516]. Homology modeling of the catalytic core of HBV polymerase revealed that the mutation at Met522 was most likely to directly contribute to this steric hindrance, where as mutation at Leu528 induced broader rearrangement of nucleotide pocket residues [516]. 3TC treatment has also been administered to reduce HTLV-1 infection and lessen the associated myelopathy exhibited by HTLV-1 infected patients [515]. For instance, in a pilot study of 5 HTLV-1 patients administered 3TC, a 10-fold reduction in viral DNA was observed [515].

The potential use of 3TC to inhibit ZEBOV replication or treat EVD patients in an emergency context remains controversial. During the 2014-16 West Africa ZEBOV outbreak, there were anecdotal reports of 3TC being used to treat patients with EVD in Liberia. For instance Dr. Logan, the Chief Health Officer of Bomi County in Liberia, administered AZT or 3TC to 15 ZEBOV patients in his care, of which 13 survived [518]. However, these results have not been published as peer-reviewed clinical case studies. For a potential mechanism of action for 3TC, 3TC-TP is predicted to interact with the putative nucleotide binding site of ZEBOV RdRP L, comparable to FPV-RTP, BCX4430-TP and AZT-TP [456]. Modeling of 3TC-TP docking
suggests it would bind this region from the “left” side, resembling FPV-RTP and BCX4430-TP binding [456]. The interaction energy of 3TC-TP with the polymerase was comparatively higher (-7.26 kcal/mol) than other nucleotide analogues modeled in silico, suggesting weaker competition with natural NTP binding would be predicted [456]. Indeed, the effects of 3TC in a preliminary cell-based assay did not show activity in reducing ZEBOV-Kikwit infection in Vero E6 cells, Hep G2 cells, or in human monocyte-derived macrophages [519]. However, these experiments tested 3TC treatment 1 hr prior to infection, which may not be enough time for cellular enzymes to convert the prodrug to active 3TC-TP. It has been reported in human subjects that at 3TC oral doses of 150 or 300 mg, peak intracellular concentration of 3TC-TP in PBMCs occurred at 2.95 and 4.10 hours, respectively [415]. Moreover the in vitro ZEBOV study of 3TC did not include a nucleoside analogue prodrug as a +ve control for ZEBOV inhibition, but rather the drug toremifene (TOR), which destabilizes the ZEBOV GP1,2 trimer and triggers early release of GP2, [519, 520]. Already in its active form, TOR would be expected to show antiviral activity 1 hr prior to ZEBOV infection [519]. Accordingly, these disparate findings that predict 3TC interacting with the ZEBOV nucleotide binding pocket [456], weak in vitro inhibition of ZEBOV replication [519], and use to treat some EVD patients in Liberia [518], warrant further in vitro testing of 3TC during ZEBOV replication, to clarify potential antiviral effects of this drug.

**Tenofovir (TFV)**

Tenofovir disoproxil fumarate (TFV-DF) is an adenosine monophosphate nucleotide analogue, belonging to the same class of phosphonates as BCV and CDV [521]. While TFV cannot cure HIV-1 or HBV infection, it is used in combination with other drugs to prevent or manage HIV-1, and ameliorate chronic HBV symptoms [522, 523]. After oral administration, TFV disoproxil fumarate becomes absorbed in the gut and cleaved, releasing TFV into the bloodstream [521]. The phosphonate moiety of TFV allows it to be rapidly mono- and diphosphorylated to the active drug TFV-DP in two steps, rather than three. TFV-DP inhibits HIV-1 RT activity through premature cDNA chain termination, and shows limited inhibition of human DNA polymerase α, β, γ [521, 524]. While viral RNA and DNA polymerases discriminate between NTPs and dNTPs
during RNA or DNA chain elongation, this does not occur with TFV-DP because of its absence of a ribose or deoxyribose moiety in its bioactive structure [456].

In 1998, it was discovered that TFV selectively inhibits HIV-1 reverse transcriptase [521]. TFV alone, or in combination with rilpivirine (RPV) and emtricitabine (FTV), can form dead-end complexes with the RT polymerase of HIV-1, producing chain-terminated DNA primer/template [525]. This effect was synergistically enhanced by the presence of the other two NRTI drugs, leading to more stable chain termination and inhibition of HIV-1 replication [525]. TDF treatment in HIV-1 patients has been successful when they harbor HIV-1 quasispecies with substitution mutations that provide resistance to multiple NRTIs, described as the Q151M complex [526]. This multidrug-resistant HIV-1 genome carries five characterisitic mutations in the RT gene: A62V, V75I, F77L, F116Y and Q151M [526]. However, it has recently been found in an HIV-1 clinical isolate that an additional RT mutation, K70Q, can add a high level of HIV-1 resistance to TFV treatment, and only in viruses that already have the Q151M complex background [526]. The additional K70Q mutation does not confer increased excision of TFV from the cDNA chain, but changes hydrogen bonding patterns in the polymerase [526]. This selectively reduces the affinity of TFV-DP to the nucleotide binding pocket of HIV-1 RT, reducing TFV incorporation into the newly synthesized cDNA chain [526]. Another important HIV-1 RT resistance mutation to TFV is K65R, however there is a low prevalence of sexual transmission of various HIV-1 subtypes carrying this substitution mutation [527]. This finding has important implications for the inclusion of TDF in PrEP regimens, as TDF reduces the transmission of HIV-1 to uninfected people [527].

The interactions of TFV with the RT of HBV have also been extensively studied. In an open-label study of twenty HIV-1 and HBV coinfected patients, a significant decrease in HBV viral load was measured after 52 weeks of daily TFV treatment, as part of their cART [522]. This was true even for 11 coinfected patients with HBV carrying the 3TC resistance sequence YMDD or YIDD [522]. Unique to HBV DNA polymerase, its own protein sequence can initiate viral cDNA synthesis as a protein primer [528]. It has been shown that TFV-DP strongly inhibits the second stage of protein priming, becoming incorporated into the viral DNA primer instead of dAMP [528]. From 2008 until 2014, no TFV drug resistance mutations have been detected in
clinical samples of HBV from patients treated with TFV-DF monotherapy [529]. To explain this phenomenon, *in silico* modeling of the HBV polymerase suggests TFV has significantly strong binding affinity to the nucleotide pocket of this enzyme [529]. In the HBV genome, the polymerase gene overlaps another gene coding for surface protein. This creates an additional genetic constraint that limits the mutational freedom that would allow for TFV drug resistance to develop during HBV infection [529].

Little preclinical research has evaluated the potential antiviral activity of TFV or TFV-DF against RNA viruses, including ZEBOV. Of the 20 nucleotide analogues modeled to dock to the putative nucleotide binding domain of ZEBOV RdRP L, TFV-DP had the lowest total interaction energy (-12.51 kcal/mol) [456]. TFV-DP was the best nucleotide/nucleoside candidate predicted to outcompete natural NTPs binding the FLRQIVRR motif [456]. Similar to AZT-TP, TFV-DP was modeled to bind this motif from the “right” side [456]. Given the absence of a ribose moiety in the structure of TFV, whether TFV is capable of inhibiting the RdRP L of ZEBOV or ZEBOV replication in living cells, deserves further attention [456].

**Combination regimens to inhibit ZEBOV**

When infected healthcare workers were airlifted from sites of ZEBOV exposure in West Africa to be treated in Western hospitals, these patients had a collectively low mortality rate [25]. This lower rate might be attributed to the multiple therapeutics administered to these patients, in addition to intensive supportive care [431, 443, 451]. Thus, research into therapy combinations that inhibit ZEBOV replication, such as ZMapp or TKM-Ebola formulations, are a high priority in developing an effective ZEBOV treatment [21, 30]. *In vitro* and small animal model research into combinations of IFNs with ZMapp, which may synergistically inhibit ZEBOV replication, are underway [6]. Furthermore the JIKI trial authors surmised that in addition to optimization of the FPV dosing regimen, the possibility of treatment combinations with FPV should also be evaluated in future EVD clinical trials [6, 18]. This led to oral FPV and ZMapp combinations being administered in the randomized, controlled trial PREVAIL II, which had sites in Libera, Guinea, Sierra Leone and the United States [19]. In addition, low doses of the nucleoside analogue ribavirin have shown to potentiate the activity of FPV in guinea pigs challenged with
Junin virus, the (-)ssRNA arenavirus responsible for Argentine hemorrhagic fever [435]. FPV and ribavirin synergistically inhibit a variety of other (-)ssRNA viruses that cause hemorrhagic fevers: Pichinde virus in hamsters [435], Lassa virus in cell culture and in lethal mouse model of infection [530], and mice infected with Rift Valley Fever Virus (RVFV) [436]. Hence, drug combinations that improve the efficacy of FPV are of great interest in ZEBOV preclinical and clinical research.

The molecular docking model of the ZEBOV nucleotide binding pocket provides additional rationale for considering two-drug combinations for inhibition of ZEBOV replication in vitro [456]. When nucleoside analogues preferentially dock on the “left” side of the nucleotide binding motif (FPV-RTP, BCX4430-TP and 3TC-TP), it leaves the “right” side open for binding of ATP, CTP, GTP, and UTP can still bind from the “left” side (see Figure 1.16, from [456]). Likewise, nucleotide analogues predicted to bind the “right” side of the nucleotide binding motif (AZT-TP and TFV-DP), leave entry on the “left” side for ATP, CTP, GTP and UTP binding [456]. Thus, optimal inhibition of the nucleotide binding pocket predicts a combination of two drugs that flank both sides of the motif, where one preferentially occupies the “left” and the other inhibitor occupies the “right” side, to sufficiently outcompete incoming NTPs [456].

Recalculating the interaction energy in the scenario of two different nucleotide analogues binding the enzyme, such as FPV-RTP + TFV-DP, considerably lowers their total interaction energy [456]. It becomes -12.28 kcal/mol for FPV-RTP and -10.80 kcal/mol for TFV-DP, much lower than the values for ATP (-4.84 kcal/mol), CTP (-6.83 kcal/mol), GTP (-7.55 kcal/mol) or UTP (-8.11 kcal/mol) [456]. A reduction of 1 kcal/mol, indicates a 10-fold higher affinity for binding, in a simple E + S⇌ES model [456]. Thus, the interaction energies of natural NTPs for RdRP L are significantly surpassed by specific combinations of two nucleotide analogues, which specifically occupy both flanking sites of the nucleotide binding pocket [456]. Administration of FPV + TFV for example, may inhibit ZEBOV polymerase activity and subsequent transcription and replication of the viral (-)ssRNA genome. This strategy has two additional benefits over monotherapy: 1) It could impede the rate of selection for drug resistant mutations against both drugs, and 2) Inhibiting viral replication in vivo during the window period of high viremia days
Fig. 1.16: Nucleotide analogues binding ZEBOV RdRP L in the presence of NTPs. (A) Abacavir-TP (red) docks to the nucleotide binding motif from the “left”, allowing most NTPs (green) access to the “right” side. (B) The opposite trend is found for tenofovir-DP (red), where it docks from the “right”, permitting NTPs to bind at the “left” [456].
after infection [18, 23], could provide the immune system enough relief to mount an effective adaptive response to clears ZEBOV infection [456].

Treating retrovirus or RNA virus infections with specific combinations of nucleoside analogues was a paradigm shift in 1996, ushering in the era of combination antiretroviral therapy for treating HIV-1/AIDS [531]. Drug synergy can help lower plasma viremia by inhibiting multiple viral targets, reduce drug toxicity by requiring lower drug doses, and provide a higher barrier to selecting resistance mutations that occur with monotherapy, as previously described for HIV-1, HBV and HCV [178, 481, 532, 533]. While routinely used to treat HIV-1, HBV or co-infection of both viruses [410], cART has not yet been explored in preclinical ZEBOV research. This is partly due to constraints caused by limited access to BSL4 facilities for in vitro or animal testing. In 2014, a sophisticated new trVLP model of the ZEBOV replication lifecycle was developed, allowing for rapid testing of therapeutics targeting any stage of the virus lifecycle in a BSL2 laboratory [534, 535]. This permitted efficient testing of drug combinations that was not previously feasible. Furthermore, the potential mechanism of action of AZT, 3TC or TFV during ZEBOV replication remained untested in living cells. Nucleoside analogues can disrupt viral replication by interfering with polymerase NTP binding [506], induce RNA chain termination [417, 426], or increase lethal mutagenesis [536], among other mechanisms [488]. Additionally, it has not been tested whether 3TC, AZT or TFV potentially synergize with leading ZEBOV drugs candidates such as FPV, fast-tracked by the WHO for phase II/III EVD clinical trials.
1.4 Statement of the Problem, Rationale, Hypotheses and Objectives of this Work

1.4.1 Statement of the Problem

Discovering host non-receptor tyrosine kinases that facilitate HIV-1 infection, yet lessen replication when inhibited with small-molecule inhibitors, is an underexplored area of research that could improve conventional cART. Moreover, with no approved treatments for EVD, repositioning drugs that directly inhibit ZEBOV replication, such as nucleoside analogues that inhibit DNA or RNA synthesis of other viruses, may inhibit ZEBOV replication in vitro.

1.4.2 Rationale

Both c-SRC and PTK2B have been implicated during HIV-1 infection from various means of investigation. Shortly after TCR-CD3 activation of a CD4+ T-cell, or G protein-coupled receptor stimulation, both c-SRC and PTK2B interact and become activated [197, 293, 340]. In addition, c-SRC and PTK2B also become activated after treating immature DCs with HIV-1 gp120 [323], or infection of Jurkat T-cells with intact virus [214]. HIV-1 also rearranges the cytoskeleton upon fusion and entry, and PTK2B has defined roles in T-cell cytoskeletal rearrangements at focal adhesions [340]. Moreover JCaM1.6 T-cells, which lack functional LCK and FYN expression but expresses c-SRC, are more infectable with HIV-1 [209]. Inhibition of the SFKs in CD4+ T-cells with PP2 inhibited viral p24 production after 6 days [264], while shRNA knockdown of PTK2B in T-cell lines reduced HIV-1 infection after 6 days [345]. HIV-1 Nef binds and activates c-SRC kinase activity [320], and may be the cause of Nef-induced AIDS phenotype in transgenic mice [164, 168]. In addition, proteins downstream of c-SRC signaling (SAMHD1 in the cytosol, Sam68 in the nucleus) already have defined roles during HIV-1 infection [317, 537]. Furthermore, c-SRC does not contribute to Gag-mediated viral assembly during budding [227], suggesting the effects of c-SRC signalling may occur at earlier stages of the viral lifecycle. My thesis will attempt to answer the following questions: Are c-SRC and PTK2B natural restriction factors of HIV-1 during early T-cell infection, or do they facilitate
early HIV-1 infection in T-cell lines and primary CD4+ T-lymphocytes? At which stages of the viral replication cycle do c-SRC and PTK2B have an effect? And if one or both facilitate infection, can FDA-approved small-molecule kinase inhibitors that target either of these NRTKs, be used to inhibit HIV-1 infection in primary CD4+ T-cells?

My thesis will also make use of a new trVLP model of ZEBOV replication, permitting rapid assessment of new therapies and combinations of therapies that can be tested in a BSL2 lab environment [534, 535]. The nucleoside analogues BCV, FPV, BCX4430 and GS5734 demonstrate broad-spectrum inhibition of other RNA viruses, DNA viruses and retroviruses [6, 357, 417, 455], becoming fast-tracked by the WHO for additional ZEBOV preclinical research [28, 418, 441], emergency phase II/III efficacy trials in West Africa [18, 24], and ZEBOV post-exposure treatment [427, 443, 450]. Other nucleoside analogues AZT, 3TC and TFV, which show potent inhibition of HIV-1, HBV and other (-)ssRNA viruses that cause hemorrhagic fever [479, 493, 497, 505, 514, 521], have been predicted to bind the ZEBOV nucleotide binding motif of RdRP L, with similar or greater affinity than natural NTPs or other nucleotide analogues modeled [456]. Moreover, combinations of nucleotide analogues that preferentially occupy the “left” side of the nucleotide binding pocket (FPV-RTP, BCX4430-TP and 3TC-TP) are predicted to synergize with drugs that enter the “right” side of the binding pocket (AZT-TP and TFV-DP) [456]. AZT, 3TC and TFV show limited inhibition of human DNA polymerases, and broad biodistribution to immune cells and tissues where ZEBOV replication persists during acute and chronic infection [416, 484, 508, 538]. Furthermore, anecdotal reports of patients treated for EVD with 3TC and AZT in Liberia warrant further testing of these drugs in cell culture infection of ZEBOV [518]. Combination regimens with FPV, the most promising drug candidate in ZEBOV clinical trials to date [18, 19], and other experimental therapies such as IFN treatment, should also be compared to one another to prioritize which combinations are tested against fully infectious ZEBOV in BSL4.

1.4.3 Hypotheses

1) Inhibiting or reducing host non-receptor tyrosine kinases c-SRC or PTK2B will restrict early HIV-1 infection in T-cell lines or primary human CD4+ T-cells.
2) Nucleoside/nucleotide analogue inhibitors AZT, 3TC and TFV, which demonstrate potent antiviral activity against other RNA viruses and retroviruses, will inhibit an *in vitro* model of ZEBOV replication alone or in combination with other therapies.

### 1.4.4 Objectives of this Work

1) Assess whether c-SRC and PTK2B facilitate HIV-1 infection in T-cell lines, by inhibiting or reducing their expression with ADV vectors expressing dominant-negative kinase mutants, tool-drug inhibitors or siRNA knockdown.

2) Determine whether c-SRC and PTK2B have similar roles in primary CD4+ T-cells collected from healthy human donors, and investigate whether FDA-approved SFK inhibitors, or SFK inhibitors in advanced clinical trials, can inhibit HIV-1 replication *ex vivo*.

3) Evaluate whether antivirals with established safety profiles and efficacy in inhibiting other RNA viruses or retroviruses, can inhibit ZEBOV replication alone or in combination, in an *in vitro* trVLP model of infection or with fully-infectious ZEBOV.

### 1.4.5 Organization of the Thesis

The strong evidence of the SFK roles during the HIV-1 replication cycle, coupled with new kinase inhibitors with enhanced target specificity being evaluated in clinical trials outside of cancer research, motivated the investigations contained in this thesis. Moreover drug repurposing and combination therapy with nucleoside analogues, successful at treating HIV-1 or HBV infection, have not been adequately investigated in ZEBOV replication *in vitro*.

In chapter 2, “c-SRC and PTK2B Protein Tyrosine Kinases Play Protective Roles in Early HIV-1 Infection of CD4+ T-Cell Lines,” kinase inhibitors, protein overexpression ADV vectors and siRNA knockdown are used to determine the roles of c-SRC and PTK2B during *in vitro* HIV-1 infection in BSL3 containment. These T-cell line experiments laid the groundwork towards a more comprehensive study of both kinases during HIV-1 replication in primary human CD4+ T-cells in chapter 3; “c-SRC Protein Tyrosine Kinase Regulates Early *HIV*-1 Infection Post-Entry.”
This chapter investigates four c-SRC inhibitors (FDA-approved or under clinical investigation) for their ability to inhibit HIV-1 infection ex vivo, using X4 and R5 lab-adapted strains and clinical isolates of HIV-1. It was also determined at which early stage during the viral lifecycle that is most dependent on c-SRC or PTK2B kinase expression.

In chapter 4, “A Rapid Screening Assay Identifies Monotherapy with Interferon-β and Combination Therapies with Nucleoside Analogues as Effective Inhibitors of Ebola Virus,” nucleoside/nucleotide analogue inhibitors were assessed in a new model system of ZEBOV replication in vitro. Timely during the 2014-16 West African ZEBOV outbreak, nucleoside analogues and therapeutics fast-tracked for EVD clinical trials were compared or combined in vitro, to assess whether they inhibit ZEBOV transcription and replication competent virus-like particles, or fully-infectious virus tested under BSL4 conditions at the National Microbiology Laboratory in Winnipeg.

Lastly in chapter 5, “Key Findings, Future Perspectives, Conclusions and Broader Significance,” limitations of the present work are discussed, as well as how the findings of this thesis relate to preclinical HIV-1 and ZEBOV drug discovery. This chapter also suggests future studies that could investigate promising therapeutics evaluated in this thesis, to potentially inhibit viral replication in animal models of HIV-1 or ZEBOV infection.
Chapter 2: c-SRC and PTK2B Protein Tyrosine Kinases Play Protective Roles in Early HIV-1 Infection of CD4+ T-Cell Lines.

Role of author McCarthy S.D.: Performed the experiments for Figure 2.1B, and all the experiments for Figures 2.2-2.7. I analyzed all the data, wrote the manuscript draft, and performed experiments to address reviewer’s comments.

A version of this chapter was published in the Journal of Acquired Immune Deficiency Syndromes:

2.1 Abstract

**Background:** During early HIV-1 infection of CD4+ T-lymphocytes, many host protein tyrosine kinases (PTK) become activated within minutes, including phosphoprotein pp60c-SRC (c-SRC) and the focal adhesion kinase family member, protein tyrosine kinase 2 beta (PTK2B, Pyk2). Whether their activation facilitates or impedes infection remains to be determined.

**Methods:** c-SRC kinase inhibitors (SU6656, PP1 and PP2), adenovectors (wild type (WT) and dominant-negative (DN) c-SRC) or siRNA (targeting c-SRC or PTK2B) were used to inhibit, compete with or knockdown c-SRC in Jurkat C, Jurkat E6-1, Hut 78 or Kit225 T-cell lines. Cells were then infected with HIV-1 luciferase reporter virus expressing VSV-G or HXB2(X4) envelope and luciferase activity was measured after 2 days. Reverse transcriptase activity and viral cDNA were measured 1 hr post-infection, while integrated virus was measured 12 hr post-infection.

**Results:** Pre-treating Jurkat T-cells with SU6656 led to increased VSV-G luciferase activity. In the adenovector experiments, T-cells overexpressing DN c-SRC, but not WT c-SRC, showed increased luciferase activity following VSV-G infection. siRNA knockdown of c-SRC or PTK2B, followed by HXB2 infection in Jurkat T-cells, lead to increased reverse transcriptase activity, viral cDNA, integrated virus, as well as increased luciferase activity.

**Conclusions:** PTK2B is known to interact with c-SRC. Thus PTK2B activation, which coincides with increased c-SRC activity during HIV-1 infection, could be responsible for c-SRC activation. Reduced c-SRC activation increases HIV-1 reverse transcription, integration and/or transcription, suggesting the high c-SRC activity seen early in HIV-1 infection may be a cellular response to slow or prevent early infection in CD4+ T-cells.
2.2 Introduction

Chronic immune activation and T-cell dysregulation persists during asymptomatic HIV-1 infection, and yet we still do not have a clear understanding of the early stages of these events [83]. From the initial binding of viral glycoprotein 120 (gp120) to T lymphocyte receptor CD4 and chemokine coreceptors CXCR4 or CCR5, signaling cascades are induced that promote viral infection [147]. It is known that tyrosine kinases are major regulators of HIV-1 infection because the pan-tyrosine kinase inhibitor, genistein, inhibits HIV-1 infection in macrophages [151]. Moreover, HIV-1-infected patients show defective early protein tyrosine phosphorylation in peripheral blood mononuclear cells [149] and in CD4+ T-cells specifically [148]. Indeed, much research has been done on the proximal signaling cascades induced upon viral binding and fusion, which interfere with normal T-cell activation and cortical actin rearrangement [539]. However, a functional role for many kinases hijacked immediately following viral entry remains to be discovered.

Non-receptor tyrosine kinases play critical roles throughout the HIV-1 lifecycle in T-cells. Changes in tyrosine phosphorylation signaling are necessary for viral entry [209], actin remodeling [135, 539], viral RNA reverse transcription [135], translocation of the viral pre-integration complex (PIC) to the nucleus, viral integration [155], viral DNA transcription and viral egress [152]. In particular, HIV-1 infected T-cells show striking changes in the activity of the SRC-family of tyrosine kinases [214]. They become activated within minutes of HIV-1 infection; however their roles at this early time point are only partially understood. There are eight SRC family members, four of which are expressed in T-cells: LCK, c-SRC, FYN and c-YES. LCK has been the most studied of these four in terms of early HIV-1 infection [148, 209, 217], as it is a proximal signaling molecule directly associated with CD4 and is critical for T-cell activation and growth [213]. Increased LCK activity was found to reduce viral replication in various T-cell lines [209]. In addition, increased FYN activity correlates with greater recruitment of tyrosine-phosphorylated APOBEC3G into HIV-1 particles [238], corroborating our previous observation of higher FYN activity in patients with asymptomatic HIV-1 infection.
Clearly the SRC family of protein kinases is implicated in HIV-1 infection, but the functional role of activated c-SRC during early HIV-1 entry remains unknown.

Previously we have shown that negligible c-SRC protein exists in resting peripheral blood T lymphocytes, but that it is produced within 12 hours of T-cell stimulation [293]. c-SRC phosphorylates more than 64 known cellular substrates, activating signaling cascades involved in cell migration, cytoskeletal rearrangement, cell proliferation and cell survival [294]. The enzyme often associates with cell membranes, in particular at focal adhesions of the plasma membrane and the perinuclear golgi region [189]. When HIV-1 initially attaches to the host membrane, viral gp120 is able to bind the G protein-coupled receptor CXCR4 or CCR5, inducing the autophosphorylation of protein tyrosine kinase 2 beta (PTK2B, Pyk2) within the cell [147]. PTK2B plays an important role at focal adhesions at the cell periphery and in cytoskeleton remodeling. Activated PTK2B can then recruit the binding of the c-SRC SH2 domain, which allows c-SRC to be autophosphorylated at Tyr419, activating the enzyme [197, 322]. Both of these kinases become phosphorylated within minutes of HIV-1 infection. Recent work has shown that PTK2B shRNA knockdown in T CEM cells reduced viral p24 production within six days of HIV-1NDK infection [345]. Moreover Gilbert et al. showed that inhibiting c-SRC with the kinase inhibitor PP2 caused a dramatic decrease in p24 production after six days of infection [264]. However, the roles of PTK2B and c-SRC in the first few hours of early HIV-1 infection, when these enzymes are first activated, are not well defined.

We hypothesize that c-SRC and PTK2B non-receptor tyrosine kinases play an important role in facilitating early HIV-1 infection of CD4+ T-cells. By using c-SRC drug inhibitors, a dominant negative c-SRC construct and siRNA knockdown, we were surprised to find that reducing c-SRC protein levels or its activity caused an increase in viral infection in three laboratory T-cell lines. This research is significant because small molecular kinase inhibitors targeting c-SRC may not be efficacious in reducing initial HIV-1 infection, but may be valuable in studying the formation and/or stability of the HIV-1 reverse transcriptase complex and pre-integration complex.
2.3 Materials and Methods

**Cells and Cell Culture Conditions:** In these *in vitro* experiments, we used the human T-cell leukemia cell lines Jurkat C (from Jurkat-FHCRC, a gift from Dr. G. B. Mills, MD Anderson Cancer Center, Houston, TX, USA), Jurkat E6-1 and a cutaneous T lymphocyte cell line Hut 78 from the American Type Culture Collection (ATCC, Rockville, USA). We also used a chronic lymphocytic leukemia T-cell line, Kit 225, also a gift from Dr. G. B. Mills. These T-cell lines were grown in complete medium consisting of RPMI-1640 with L-glutamine and NaHCO₃ (SIGMA, St. Louis, USA), supplemented with 10% (vol/vol) heat-inactivated Fetal Bovine Serum (FBS; Wisent, Saint-Jean-Baptiste, Canada), 100 units/mL penicillin (Gibco, Burlington, Canada), 100 μg/mL streptomycin (Gibco), 10 μg/mL gentamycin (Gibco), and 10 units/mL human recombinant IL-2 (Kit 225 cells only; SIGMA). Human embryonic kidney cells (HEK 293T, ATCC) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) with the same proportion of FBS and antibiotics as above. MT-4 cells from the NIH AIDS Research and Reference Reagent Program (Germantown, USA) were used to determine viral multiplicity of infection (MOI). All cells were incubated at 37°C in a 5% CO₂ atmosphere.

**Generation of infectious HIV-1, VSV-G pseudoenveloped and HXB2 enveloped virus:** In brief, X4 HIV-1ΔIIIb from the NIH AIDS Research and Reference Reagent Program (Rockville) was grown in Jurkat C cells (1 x 10⁶ cells/mL) in a biosafety level 3 (BSL3) lab [540]. The cell supernatant was collected, viral stocks were titrated and assessed by p24 Antigen ELISA (ZeptoMetrix, Buffalo, USA), and aliquots were frozen at -80°C. MOI was determined using MT-4 cells [540].

We produced recombinant, replication deficient VSV-G pseudoenveloped or HXB2 enveloped (X4) HIV-1 containing a luciferase gene, as described previously [541]. Briefly, 2.5 x 10⁶ HEK 293T cells were plated with 10 mL of DMEM 24 hours prior to transfection. We co-transfected 15 μg of HIV-1 NL4-3luc (luciferase gene inserted into viral nef gene and env deficient; a kind gift from Dr. Veneet KewalRamani, New York Medical University, NY) with 10 μg of VSV-G env plasmid (amphotropic envelope of the vesicular stomatitis virus; a generous gift from Dr. M. Tremblay, Quebec City, PQ) into each 10 cm plate of HEK 293T cells with the CalPhos™
Mammalian Transfection Kit (Clontech, Mountain View, USA), following the manufacturer’s instructions. The same was performed for the HXB2 plasmid (HIV-1 NL4-3luc lacking nef and containing HXB2 (X4) env; another generous gift from Dr. M. Tremblay) at 15 μg per plate. DMEM media was replaced after 25 hours and viral supernatant was collected on days 2 and 3. The supernatant was syringed through 0.45 μm pore filters (Millipore, Billerica, USA), then concentrated by underlaying with a 20% sucrose gradient and centrifuging at 16,000 x g for 90 minutes at 4 °C. Virus pellet were resuspended in TNE buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA and 100 mM NaCl) and stored in -80°C aliquots. p24 levels were measured by ELISA. All viruses underwent one freeze-thaw cycle prior to infection studies.

**c-SRC Drug Inhibition:** Prior to infection, 2 x 10⁶ T-cells were inhibited with 5-20 μM of SU6656 (In Solution™), 10 μM PP1 (Analogue) or 10 μM PP2 (Calbiochem, La Jolla, USA) for 1 hr, washed with Dulbecco’s Phosphate Buffered Saline (PBS) lacking calcium chloride and magnesium chloride (SIGMA), resuspended in RPMI media, and then counted again. The solvent Dimethyl Sulfoxide (DMSO, Bioshop, Burlington, Canada) was used as a -ve control for SU6656.

**Kinase Assay:** Jurkat C cells treated for 1 hr with SU6656 were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM HEPES, pH 7.3, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM Na₂VO₄, 50 mM ZnCl₂, 2 mM EDTA and 2 mM PMSF), and immunoprecipitated with anti-c-SRC clone 327 (Oncogene, Cambridge, USA). Lysates and beads were washed then re-suspended in kinase buffer, including 5 μCi γ³²P-ATP (MP Biomedicals, Santa Ana, USA) and heat inactivated enolase (MP Biomedicals) as substrate. Protein samples were boiled for 10 minutes and then separated on a 12% reducing SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore). Enolase phosphorylation by c-SRC was determined by autoradiography. *In vitro* kinase activity was measured as the densitometry of enolase activity divided by the amount of c-SRC per lane, and normalized to the DMSO control treatment.

**c-SRC Adenovirus Gene Transduction:** Recombinant Ad5/F35 adenovirus vectors were created by Daniel Jung. These bicistronic vectors contained enhanced yellow fluorescent protein
(EYFP), and expressed wild type human c-SRC (WT), a dominant negative, kinase inactive c-SRC (DN) or no c-SRC as a control (empty vector; EV) [190]. Cells were titrated with virus to determine the highest multiplicity of infection that permitted 70-80% gene transduction, while minimizing cell death 48 hours post-infection. EYFP detection and viability staining (7-AAD, which stains both apoptotic cells and necrotic bodies, BD Sciences, Franklin Lakes, USA) were performed on fixed cells using FACS (see flow cytometry and data acquisition).

**siRNA Knockdown:** Pools of four siRNA duplexes, specific for human c-SRC (cat # M-003175-03) or PTK2B (cat # M-003164-02) mRNA, and a control pool of non-targeting siRNA (cat # D-001206-13-05) were ordered from Dharmacon RNAi technologies (siGENOME SMART Pool, Thermo Scientific, Lafayette, USA). In Jurkat E6-1 cells, siRNA knockdown was achieved using the siRNA cationic lipid transfection reagent GeneSilencer® (Genlantis, San Diego, USA), following the manufacturer’s instructions. Two μL of siGuard RNase inhibitor® (Genlantis) was added to each 1 mL reaction to prevent RNAse degradation of the RNA duplexes. Upon siRNA titration, 900 nM was determined to give the optimal level of protein knockdown for both c-SRC and PTK2B, via Western blot densitometry at 48 hours.

**HIV-1 Infection and Luciferase Assay:** 1 x 10^6 T-cells pre-treated with drug, adenovector or siRNA were infected with 1.4 ng of VSV-G/HIV-1 or 7.5 ng of HXB2 at 37°C in 2 mL of growth media in 12-well plates for 2 days. Cells were counted, while others were lysed with the Luciferase Assay System (Promega, Madison, USA) or kept for Western blots.

**Antibodies/Immunoblots:** Jurkat C cells were serum-starved and then immediately following 15 minutes of HIV-IIIb infection, were washed in PBS and lysed with RIPA buffer. Monoclonal anti-phosphotyrosine (anti-ptyr) clone 4G10 (Millipore) and protein G-Sepharose beads (Santa Cruz Biotechnology, Dallas, USA) were used to immunoprecipitate phosphoproteins from 4 x 10^6 Jurkat C cell lysates.

Following VSV-G/HIV-1 or HXB2 infection, 5-10 x 10^5 T-cells were washed in PBS and lysed with RIPA buffer. After boiling samples, proteins were resolved on an 8%, reducing SDS-PAGE gel, and transferred to an Immobilon-P membrane for Western Blot detection. The blots
were blocked with 5% skimmed-milk powder and 0.5% Nonidet P-40 (BioShop, Burlington, Canada). Primary antibodies diluted in 5% milk and 0.5% Nonidet P-40, anti-\(\beta\)-actin (Sigma), anti-\(\alpha\)-tubulin (Sigma) anti-PTK2B (Transduction Laboratories) and anti-c-SRC GD11 (Millipore) were used to probe the blots. After washing, secondary goat anti-mouse HRP antibody (Bio-Rad, Mississauga, Canada) was added, followed by enhanced chemiluminescent (ECL) detection (GE Healthcare, Buckinghamshire, UK). PTK2B and c-SRC protein levels were determined by comparing their densitometry to normalizers \(\beta\)-actin or \(\alpha\)-tubulin with the Molecular Imager GelDoc XR+ Imagin System (Bio-Rad).

**DNA isolation and quantitative PCR:** Genomic and viral cDNA was isolated from \(5 \times 10^5\) Jurkat E6-1 cells, pre-treated by siRNA transfection and infected with HXB2 virus for 1 or 12 hours, using the QIAamp DNA Blood Mini Kit (Qiagen, Toronto, Canada). Samples were heated at 56°C for 2 hours in QIAamp DNA Blood Mini Kit buffer AL to deactivate viable viral particles.

The primer sets used to detect human or viral DNA (synthesized by the Center for Applied Genomics, The Hospital for Sick Children, Toronto, Canada) were as follows [159, 542, 543]: \(\beta\)-globin forward, 5'-CCCTTGGACCCAGGTTCT -3'; \(\beta\)-globin reverse, 5'-CGAGCACTTTCTTGCCATGA-3'; early reverse transcripts (early RT) forward, 5'-GTAACTAGAGATCCCTCAG ACCCTTTTAG-3'; early RT reverse, 5'- TAGCAGTGCGCCCGA-3'; late reverse transcripts (late RT) forward, 5'-CCGTCTGTTGTGTGACTCTGG-3'; late RT reverse, 5'-GAGTCCTGCGTCTGAGAGATCT-3'; genomic Alu forward, 5'-GCCTCCAAAGTGCTGGGATTACAG-3'; HIV-1 gag reverse, 5'-GCTCTCGACCCATCTCTCC-3'; HIV-1 LTR forward, 5'-GCCCTCAATAAAGCTTGCTTGA-3'; HIV-1 LTR reverse, 5'-TCCACACTGACTAAAGGGTCTGAG-3'. For each qPCR run, 50-100 ng template DNA (with the exception of the no-template control, NTC) was added to PCR tubes containing: 12.5 \(\mu\)L 2x SYBR Green PCR Master Mix (Applied Biosystems), 300-900 nM of each forward and reverse primers, and PCR grade H\(_2\)O (Roche Diagnostics, Indianapolis, USA) up to a final volume of 25 \(\mu\)L. PCR cycling parameters were as follows: initial denaturation at 95°C for 10 min; 40-50 cycles of amplification of 95°C for 15 sec, 58°C for 30 sec (\(\beta\)-globin or late RT, 54°C for early RT) and 60°C for 30 sec. To measure integrated virus by Alu-gag PCR [543], tubes were first pre-amplified with 100 nM Alu forward
and 600 nM *gag* reverse primers, with 2x SYBR Green PCR Master Mix as above, for 10 cycles of 93°C for 30 sec, 50°C for 60 sec, and 60°C for 100 sec. Nested PCR was performed on these samples by adding 300 nM each of HIV-1 LTR forward and reverse primers, with 2x SYBR Green PCR Master Mix, for 40 more cycles using the above parameters. Duplicate reactions were analyzed using the Rotor-Gene RG-3000 thermocycler (Corbett Research, Montreal, Canada). c-SRC and PTK2B siRNA-treated groups were compared to the non-targeting siRNA-treated group by the $2^{-\Delta\Delta CT}$ comparative C_T method [544].

**Flow Cytometry and data acquisition:** Cell surface expression of CD4 and CXCR4 were determined on 2.5 x $10^5$ Jurkat E6-1 cells per FACS tube, 48 hours after siRNA knockdown. Cell surface staining was performed as previously described [545]. In brief, cells were stained with 5 μL of FITC mouse anti-human CD4, 5 μL of PE mouse anti-human CXCR4 and 2.5 μL of 7-AAD, in 100 μL of FACS buffer (PBS + 2.5% FBS). Isotype control antibodies were FITC-conjugated mouse IgG1κ and PE-conjugated mouse IgG2ακ. All of these antibodies were purchased from BD Pharmingen, Mississauga, Canada. Data on 20,000 events per tube was collected using Becton Dickenson FACSCalibur (BD Calibrite; BD Biosciences). Signals were acquired for the forward scatter (FSC), side scatter (SSC), the green (FITC, EYFP), yellow (PE) and red (7-AAD) channels.

**Reverse Transcriptase Activity Assay:** Quantification of reverse transcriptase activity from infected cell lysates was performed using the SYBR Green I qPCR-based product-enhanced RT (PERT) assay [546]. Briefly, 5 x $10^5$ JE6-1 cells were infected with HXB2 virus for 1 hr, washed twice with PBS, lysed, diluted 10-fold with 10x sample dilution buffer, and given 1 μg of MS2 RNA template (Roche Diagnostics) to reverse transcribe *in vitro*. The cDNA product was measured by qPCR, and a standard curve of recombinant HIV-1 RT (Calbiochem) was used to assign enzyme activity units to reverse transcriptase from the infected cell lysate samples.

**Statistical Analysis:** Means were compared using a two-tailed, unpaired Student’s *t* test and corrected for multiple comparisons when more than two means were considered in an experiment, to minimize the probability of type 1 errors. FACS data shows the Median Fluorescent Intensity (MFI) and percent positive cells collected with CellQuest software (BD
Biosciences) and analyzed by FlowJo version X software. Comparisons of histogram EYFP expression was performed with the two sample Kolmogorov-Smirnov test. For all figures, an asterisk (*) denotes a \( p \) value < 0.05, two asterisks (**) denotes a \( p \) value < 0.01 and three asterisks (***}) denotes a \( p \) value < 0.005. Error bars shown are the standard error around the mean (SEM).
2.4 Results

**Tyrosine kinases become activated within minutes of HIV-1\textsubscript{IIIB} infection**

To confirm previous reports that non-receptor kinases become activated within minutes of HIV-1 infection, we infected serum-starved Jurkat C T-cells with HIV-1\textsubscript{IIIB}, HXB2 (X4 strains), or with VSV-G pseudoenveloped HIV-1 virus. We then immunoprecipitated protein substrates from T-cell lysates with anti-ptyr clone 4G10 (Fig. 2.1). Probing the blot of HIV-1\textsubscript{IIIB} infected cell lysate with anti-ptyr revealed a robust phosphorylation of substrates in the 55-130 kDa range, and one low molecular band at ~40-42 kDa (Fig. 2.1A). Re-probing the Western blot for PTK2B and c-SRC (Fig. 2.1B), demonstrated phosphorylation of these kinases within 15 minutes of HIV-1 infection, indicative of their activation and recruitment during early HIV-1 entry events.
Fig. 2.1: Western blot of phosphorylated proteins from serum-starved T-cells shortly after HIV-1 infection. 4 x 10^6 Jurkat C T-cells were infected with 22.8 ng of VSV-G/HIV-1, HXB2 or HIV-1_{IIIb} for 15 minutes, washed in PBS and lysed with RIPA buffer. Phosphoproteins were than immunoprecipitated using monoclonal anti-phosphotyrosine (anti-ptyr) clone 4G10, and run on a 10% reducing SDS-PAGE gel. Lane one is Jurkat C cell lysate, lane two is anti-ptyr antibody, and lane three is immunoprecipitated infected Jurkat C cell lysate. A Western blot transfer was performed and the blot was probed for total phosphoproteins with anti-ptyr clone 4G10 (Panel A), anti-PTK2B or anti-c-SRC GD11. Panel B shows the densitometry of tyrosine phosphorylated PTK2B and c-SRC in a single experiment.
SU6656 consistently inhibited c-SRC activity, and increased VSV-G/HIV-1 infection

To directly implicate a role for c-SRC activity during early HIV-1 infection, we used well known c-SRC inhibitors (PP1, PP2 and SU6656) [184] to inhibit c-SRC activity prior to HIV-1 infection. For these experiments a replication deficient, pseudotyped nef-deficient VSV-G/HIV-1 virus carrying a luciferase reporter gene was used, so as not to produce viral protein products which are known to directly bind and activate the SRC family of kinases, such as viral protein Nef [164]. After pre-treating Jurkat E6-1 cells with 10 μM of drug for 1 hour, only the SU6656 treated cells showed a significant increase in VSV/G/HIV-1 infection after two days, compared with DMSO control (Fig. 2.2A). Cell growth was not adversely affected during this experiment (Figure 2.3A). The non-specific effects of SU6656 differ from PP1 and PP2 [184], which is why we decided to move forward using SU6656 as our c-SRC inhibitor. Our in-vitro kinase assay showed that SU6656 reduces c-SRC activity by 50% at 10 μM (Fig. 2.2B). We repeated our VSV-G/HIV-1 infection experiment at two different SU6656 drug concentrations (Fig. 2.2C) and in another Jurkat T-cell line (Fig. 2.2D) to confirm our finding that c-SRC drug inhibition leads to increased VSV-G/HIV-1 infection in T-cell lines.
Fig. 2.2: Jurkat T-cell lines inhibited with SRC-family kinase inhibitors. Panel A: 2 x 10^5 Jurkat E6-1 cells were pre-treated with 10 μM of DMSO (control), PP1, PP2 or SU6656 for 1 hr, washed in PBS, then infected with 1.4 ng of VSV-G/HIV-1 for two days. Cells were then counted, while other cells were lysed to measure luciferase activity (RLU) to quantify viral infection. This experiment was repeated 3 times. Panel B: After treating 2 x 10^5 Jurkat C cells with 5 or 10 μM SU6656 for 1 hr in a single experiment, cells were lysed, c-SRC protein was immunoprecipitated, and in-vitro kinase activity was determined by the phosphorylation of enolase substrate with γ^32P-ATP. Panel C and D: 2 x 10^5 Jurkat E6-1 cells (C) or Jurkat C cells (D) were pre-treated with 10 or 20 μM of SU6656, and then infected with 1.4 ng of VSV-G/HIV-1 for two days. Luciferase activity was then measured. Standard error bars are of triplicate measurements.
Fig. 2.3: Cell survival was not significantly different between c-SRC drug or adenovirus vector treatments. **Panel A:** $2 \times 10^5$ Jurkat E6-1 cells were pre-treated with 10 μM of DMSO (control), PP1, PP2 or SU6656 for 1 hr, washed in PBS, and then grown for two days in RPMI. Cells were then counted using trypan blue stain exclusion. **Panel B:** T-cell lines were transduced with adenovectors expressing c-SRC mutants. Adenovirus vectors (EV = empty vector, WT c-SRC = Wild Type c-SRC, DN c-SRC = Dominant Negative c-SRC) were administered to Jurkat E6-1, Hut 78 and Kit 225 cells at an MOI of 750 for 48 hr. The percent of live cells (7-AAD -ve) was then measured using FACS in a single experiment.
Adenovector expressing dominant negative c-SRC increased VSV-G/HIV-1 infection in CD4+ T-cell lines

As with most kinase inhibitors, we cannot ignore that SU6656 inhibits related SRC family members, such as LCK and FYN, in conjunction with c-SRC. Thus, we used adenovirus gene transduction to alter the pool of functional c-SRC in three T-cell lines, prior to VSV-G/HIV-1 infection. Jurkat E6-1, Hut 78 and Kit 225 T-cells were transduced with adenoviruses containing an Empty Vector control (EV) Wild Type (WT) c-SRC or a Dominant Negative (DN) c-SRC that can bind but not phosphorylate substrate (Figure 2.4). Administering adenovector expressing WT c-SRC or DN c-SRC did not alter cell survival when compared to EV, within each cell type (Figure 2.3B). Once we optimized multiplicity of infection (MOI) to reduce cell death and maintain high gene transduction, we achieved 62-84% EYFP expression in Jurkat E6-1 and Hut 78 cells after 2 days (Fig. 2.4B-C). Kit 225 cells were less transducible (36-47%, Fig. 2.4D), but showed higher overall survival. No statistical difference was found between EV, WT c-SRC or DN c-SRC EYFP expression, by the Kolmogorov-Smirnov test.

Viral infection was normalized to the total cells in each well after two days of VSV-G/HIV-1 infection. At this time point, no significant difference in VSV-G/HIV-1 infectivity was found between EV and WT c-SRC treatments (Fig. 2.4E-G). However, administering the DN c-SRC adenovector caused a significant increase in VSV-G/HIV-1 infection in two of the three T-cell lines tested. This finding complements our previous c-SRC drug experiments. Competing endogenous c-SRC enzyme with the dominant negative mutant increases the integration or transcription of VSV-G/HIV-1.
Fig. 2.4: T-cell lines were transduced with adenovectors expressing c-SRC mutants. Adenovirus vectors (EV = empty vector, WT c-SRC = Wild Type c-SRC, DN c-SRC = Dominant Negative c-SRC) were administered to Jurkat E6-1, Hut 78 and Kit 225 cells at an MOI of 750 for 48 hr. FACS gating was performed on live cells that were 7-AAD negative (Panel A). On this cell subset, EYFP was measured to determine gene transduction efficiency (Panels B-D). Median fluorescent intensity (MFI) and % positive cells are shown. After two days of adenovirus gene transduction, 2 x 10^5 cells were infected with 1.4 ng VSV-G/HIV-1, and luciferase activity measured after an additional two days (Panels E-G). Standard error bars are of triplicate measurements.
Both c-SRC and PTK2B knockdown caused an increase in either VSV-G/HIV-1 or HXB2 infection.

To assess whether the upstream binding partner PTK2B mediates the effects of c-SRC during HIV-1 infection, we used siRNA to knockdown mRNA expression of either kinase in Jurkat E6-1 cells. Relative to a non-targeting siRNA control, cell proliferation was not affected by PTK2B or c-SRC knockdown (Figure 2.5A). T-cells are a challenging non-adherent T-cell line to transfect for siRNA knockdown, hence we optimized the siRNA concentration delivered and the time-point to assess protein knockdown by Western blot (Fig. 2.5B-G). Qualitatively, we found 900 nM of siRNA transfected by lipofection could reliably knockdown either c-SRC or PTK2B by 40-50%, 48 hours post-transfection (Fig. 2.6A-D).

At this time point, we then infected the Jurkat E6-1 cells with VSV-G/HIV-1 and measured infection two days later. Interestingly, both c-SRC and PTK2B knockdown in Jurkat E6-1 cells lead to an increase in luciferase activity upon infection with the VSV-G/HIV-1 reporter virus, when compared with the non-targeting siRNA control group (Fig. 2.6E). This implicates both kinases as having important catalytic activity during early HIV-1 infection of T-cells. To determine whether this phenomenon can be attributed to the differences in vesicular stomatitis entry into a host T-cell (clathrin-dependent endocytosis) [547] and that of HIV-1 (receptor-mediated internalization), we performed the siRNA knockdown experiment once more with an HXB2 (X4) luciferase reporter virus that is also replication-deficient. Similar to the VSV-G/HIV-1 infection, when either c-SRC or PTK2B protein expression is reduced by siRNA knockdown, we saw a concomitant increase in HXB2 viral infection after two days (Fig. 2.6F). This suggests that PTK2B and c-SRC may play similar roles in the early HIV-1 entry events of both viruses downstream of HIV-1 receptor binding and fusion.
Fig. 2.5: siRNA titration and time-course optimization in Jurkat E6-1 cells. Panel A: Jurkat E6-1 cell survival two days post-transfection of non-targeting, c-SRC or PTK2B siRNA, as measured by trypan blue staining. Panels B-E: Two days post-lipofection, 5 x 10^5 cells were lysed with RIPA to perform an 8% SDS-PAGE followed by a Western blot. Blots were probed with anti-c-SRC (B and C), anti-PTK2B (D and E) and anti-α-tubulin. Quantification of PTK2B and c-SRC protein was relative to α-tubulin control. Panels F and G: Time-course for optimal c-SRC knockdown post-lipofection. All of these were single experiments.
Fig. 2.6: siRNA knockdown in Jurkat E6-1 cells infected with either VSV-G/HIV-1 or HXB2 virus. Panels A and B: Two days post-transfection with non-targeting, c-SRC or PTK2B siRNA, 5 x 10^5 cells were lysed with RIPA to perform an 8% SDS-PAGE followed by a Western blot. Blots were probed with anti-PTK2B, anti-c-SRC or anti-α-tubulin. Quantification of PTK2B and c-SRC protein was relative to α-tubulin control, and normalized to the non-targeting siRNA treatment. Original Western blots are shown in Panels C and D and are representative of three independent experiments. Two days after siRNA transfection, 2 x 10^5 cells were plated and infected with 1.4 ng of VSV-G/HIV-1 (Panel E) or 7.5 ng of HXB2 virus (Panel F). Luciferase activity was measured in cell lysates 48 hr thereafter.
c-SRC and PTK2B siRNA knockdown increased the reverse transcription of HXB2 cDNA

To assess at which early stage c-SRC or PTK2B were having their effect, we measured the production of HIV-1 early reverse transcripts (early RT) late reverse transcripts (late RT), as well as integrated virus, shortly after HXB2 infection by real-time qPCR (Fig. 2.7A). One hour post-infection, both c-SRC and PTK2B siRNA knockdown led to a 4-10 fold increase in early and late reverse transcripts, relative to non-targeting siRNA treated cells. Knocking down c-SRC or PTK2B also led to a 3-4 fold increase in integrated virus measured after 12 hours of infection. We detected no increase in CD4 or CXCR4 cell surface expression after c-SRC or PTK2B knockdown (Fig. 2.7B), suggesting the increase in HIV-1 early RT, late RT and integrated virus is not due to increased receptor binding upon entry. Measuring reverse transcriptase activity 1 hour post-infection revealed increased activity from lysates of Jurkat T-cells administered c-SRC or PTK2B siRNA (Fig. 2.7C), suggesting that they act, at least in part, at the level of HIV-1 reverse transcription.
Fig. 2.7: qPCR of HXB2 cDNA extracted from Jurkat E6-1 following siRNA knockdown. Shortly after infection, early reverse transcripts (early RT) at 1 hr, late reverse transcripts (late RT) at 1 hr, and integrated virus at 12 hr post-infection were measured relative to β-globin expression (Panel A). FACS analysis of CD4 and CXCR4 expression on Jurkat E6-1 cells following 48 hr of siRNA knockdown, gating on the 7-AAD negative population (Panel B). Reverse transcriptase activity was measured from lysates of 5 x 10^5 cells after 1 hr of HXB2 infection (Panel C). Standard error bars are of five replicate measurements in A and C.
2.5 Discussion

The present study demonstrates that reducing c-SRC enzyme activity, outcompeting endogenous c-SRC with a dominant negative SRC mutant, or reducing c-SRC protein levels with siRNA knockdown caused an increase in VSV-G/HIV-1 infection in various T-cell lines. We first demonstrated that c-SRC becomes activated within 15 minutes of HIV-1\textsubscript{IIIb} infection, which agrees with our previous findings [214]. We also found that PTK2B becomes phosphorylated in the same 15 minute time span, faster than Seror et al. reported using HIV-1\textsubscript{NDK} virus [345].

Reducing c-SRC activity with SU6656 consistently caused an increase in VSV-G/HIV-1 infection in Jurkat T-cells. Initially, this finding appears paradoxical considering that CD4\textsuperscript{+} T-lymphocytes pre-treated with PP2 were previously reported to show a decrease in p24 levels six days after NL4-3bal\textsubscript{env} or JR-CSF (R5) infection [264]. This discrepancy may be because our viral read-out measured transcription of luciferase to detect successful viral integration, instead of p24 to monitor viral production and egress. The initial activation of non-receptor tyrosine kinases is rapid [214], and kinases that play a role during early entry may have different roles during viral egress and p24 release. Thus inhibiting c-SRC activity may promote early entry of HIV-1, as we have found, and yet reduce viral p24 production at later stages of the viral replication cycle. This difference in function could perhaps be attributed to the downstream substrate, the 68 kDa SRC-associated protein in mitosis (Sam 68), which is known to assist in the nuclear export and translation of HIV-1 RNA [317].

We also observed that the addition of adenovector producing dominant negative c-SRC in three T-cell lines caused an increase in VSV-G/HIV-1 infection, yet adding adenovector expressing wild type c-SRC did not reduce infection. Perhaps this can be attributed to the dynamic regulation of c-SRC by cellular phosphatases (SHP-1, CD45) and kinases (CSK), explaining why the addition of wild type c-SRC could not reduce viral infection. Nonetheless, the ability of dominant negative c-SRC to compete with endogenous c-SRC for substrates and cause an increase in VSV-G/HIV-1 infection agrees well with our drug experiments. Whether c-SRC directly phosphorylates HIV-1 proteins, or triggers signaling pathways that hinder viral entry and integration, warrants further investigation.
In terms of our siRNA knockdown findings, both c-SRC and PTK2B knockdown caused an increase in VSV-G/HIV-1 infection in Jurkat T-cells. This agrees well with the previous finding that activated PTK2B links G protein-coupled receptors to downstream signaling by complexing with c-SRC and subsequently causing c-SRC activation [197]. It is interesting to note that while VSV-G/HIV-1 enters cells via clathrin-dependent endocytosis [547], using a virus with HXB2 (X4) envelope gave a similar result, suggesting both kinases play important roles in HIV-1 infection regardless of initial entry route. We also found increased reverse transcriptase activity, early reverse transcripts, late reverse transcripts and integrated virus after c-SRC or PTK2B siRNA knockdown. PTK2B, being upstream of c-SRC, had a stronger effect on viral DNA produced in cells. It will be of interest to determine the mechanism by which these kinases affect early infection, perhaps stabilizing the reverse transcriptase complex or pre-integration complex through phosphorylation or allosteric interactions.

Our research is the first study to explore the impact of c-SRC and PTK2B activation on early HIV-1 infection in CD4+ T-cell lines. Discovering novel mechanisms in the entry of HIV-1 and their impact on T-cell signal transduction may lead to targeting these pathways with drugs and improving the treatment options available to people with HIV-1. Tyrosine kinase inhibitors with low toxicity profiles exist for cancer therapies, and could be repositioned as viable options for treating HIV-1 infection. For instance cyclin-dependent kinase inhibitors, such as r-roscovitine and alsterpaullone, are showing promising anti-HIV-1 properties in PBMCs by preventing viral Tat transactivation [548]. We also know that tyrosine kinase inhibitors can be safe in HIV-1 patients, as a case study of patients with AIDS-related Kaposi Sarcoma given the ABL kinase inhibitor imatinib showed no increase in viral plasma load [176]. Our results show increased HIV-1 early reverse transcripts, late reverse transcripts and integration when c-SRC or PTK2B are inhibited, which may preclude the use of SRC inhibitors during early HIV-1 infection. However, the potential use of c-SRC kinase inhibitors to study the stability of the reverse transcriptase complex and pre-integration complex warrants further investigation.
Chapter 3: c-SRC Protein Tyrosine Kinase Regulates Early HIV-1 Infection Post-Entry

Role of author McCarthy S.D.: Performed all the experiments for Figures 3.1-3.10 and illustrated Figure 3.11. I analyzed all the data, wrote the manuscript draft, and performed experiments to address reviewer’s comments.

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3.1 Abstract

**Objective:** We investigated whether HIV-1 inhibition by SRC-family kinase inhibitors is through the non-receptor tyrosine kinase phosphoprotein pp60\(^c\)-SRC(c-SRC) and its binding partner, protein tyrosine kinase 2 beta (PTK2B).

**Design:** CD4\(^+\) T-lymphocytes were infected with R5 (JR-FL) or X4 (HXB2) HIV-1. SRC-family kinase inhibitors or targeted siRNA knockdown of c-SRC and PTK2B were used and various stages of the early HIV-1 lifecycle were examined.

**Methods:** Four SRC-family kinase inhibitors or targeted siRNA knockdown were used to reduce c-SRC or PTK2B protein expression. Activated CD4\(^+\) T-lymphocytes were infected with recombinant, nef-deficient, or replication-competent infectious viruses. Knockdown experiments examined multiple stages of early infection by monitoring: luciferase activity, expression of host surface receptors, reverse transcriptase activity, p24 levels and qPCR of reverse transcripts, integrated HIV-1 and 2-LTR circles.

**Results:** All SRC-family kinase inhibitors inhibited R5 and X4 HIV-1 infection. Neither c-SRC nor PTK2B siRNA knockdown had an effect on cell surface receptors (CD4, CXCR4, CCR5) nor on reverse transcriptase activity. However, using JR-FL both decreased luciferase activity while increasing late reverse transcripts (16-fold) and 2-LTR circles (8-fold) while also decreasing viral integration (4-fold). With HXB2, c-SRC but not PTK2B siRNA knockdown produced similar results.

**Conclusions:** Our results suggest c-SRC tyrosine kinase is a major regulator of HIV-1 infection, participating in multiple stages of infection post-viral entry: Reduced proviral integration with increased 2-LTR circles is reminiscent of integrase inhibitors used in combination antiretroviral therapies. Decreasing c-SRC expression and/or activity provides a new target for antiviral intervention and the potential for repurposing existing FDA-approved kinase inhibitors.
3.2 Introduction

To overcome HIV-1 drug resistance, targeting host T-cell factors critical to HIV-1 infection has become a promising field of research [146]. Therapeutics designed to target host proteins are being explored in conjunction with combination antiretroviral therapy (cART) to reduce the size of the initial HIV-1 reservoir [549] or reactivate then purge HIV-1 in the cells of infected people [550]. For example, some drugs disrupt allosteric interactions between viral integrase and host proteins LEDGF/p75 [551], and other therapeutics can inhibit histone deacetylases to reactivate provirus in latently infected cells [552]. However, inhibition of tyrosine kinases activated during early HIV-1 infection remains an underexplored area of antiretroviral drug discovery. From initial binding of viral glycoprotein 120 (gp120) to the T-lymphocyte receptor CD4 and chemokine co-receptor CXCR4 (X4 viruses) or CCR5 (R5 viruses), tyrosine kinases are induced that promote viral infection [147]. Moreover, non-receptor tyrosine kinases play critical roles during the early stages of the HIV-1 lifecycle in T-cells (reviewed in [146]). Tyrosine phosphorylation signaling is necessary for viral entry [135], actin remodeling [539] and translocation of the viral pre-integration complex (PIC) into the nucleus [154, 158]. In particular, HIV-1 infected CD4+ T-cells show striking changes in the activity of the SRC-family of tyrosine kinases, which become activated within minutes of HIV-1 infection [214]. Previous work suggests that dasatinib, a dual SRC/ABL ATP competitive inhibitor, approved for the treatment of imatinib-resistant chronic myeloid leukemia [553], effectively inhibits HIV-1 production in activated CD4+ T-cells isolated from chronically infected HIV-1 patients [554]. However, a specific role for c-SRC at earlier time points, post initial infection up through integration, has not been examined.

When HIV-1 initially attaches to the host T-cell membrane through its interaction with cell-surface CD4, viral gp120 then binds the G protein-coupled receptor CXCR4/CCR5, inducing the autophosphorylation of protein tyrosine kinase 2 beta (PTK2B, Pyk2 or FAK2) within the cell [147]. PTK2B plays a key role in focal adhesions at the cell periphery and in cytoskeleton remodeling [340]. Phosphorylation of PTK2B at Tyr402 can then recruit c-SRC (SRC, pp60c-SRC) to its SRC-homology-2 (SH2) binding domain, permitting c-SRC to autophosphorylate at
Tyr419, activating c-SRC [197, 322]. Both of these kinases become phosphorylated within minutes of HIV-1 infection [147, 214].

Previously, we have reported that inhibiting or reducing PTK2B or c-SRC in various T-cell lines increased HIV-1 reverse transcription and integrated provirus [555]. However, another study has shown that PTK2B shRNA knockdown in the T CEM cell line reduced viral p24 production after six days of HIV-1NDK infection [345]. In addition, Gilbert et al. have demonstrated that inhibiting c-SRC with the kinase inhibitor PP2 decreased p24 production in primary CD4+ T-cells after six days of infection [264]. The discrepancy of PTK2B and c-SRC inhibition that promotes early HIV-1 entry within hours, yet restricts virion maturation and p24 release within days, remains unresolved. One possibility is that PTK2B and c-SRC function differently during early HIV-1 entry in activated primary CD4+ T-lymphocytes than in immortalized T-cell lines. To test this possibility, we hypothesized that inhibiting the non-receptor tyrosine kinases PTK2B and c-SRC will restrict HIV-1 early entry in activated primary CD4+ T-cells.

We demonstrate that reducing c-SRC expression with siRNA restricted the infection of X4-tropic HXB2 or R5-tropic JR-FL, Ba-L or KNH1207, within 12 hrs of infection. Interestingly, PTK2B siRNA knockdown only reduced early infection of R5-tropic viruses, suggesting different dependence on downstream signaling contingent on the co-receptor engaged during virus binding and fusion. Our findings not only provide the rationale for exploring the use of drugs to inhibit these two kinases as novel means to inhibit HIV-1 replication, but they may also suggest a unique method for arresting HIV-1 at the stage of pre-integration complex (PIC) formation and/or integration.
3.3 Materials and Methods

**Cell Isolation and Culture Conditions:** Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of healthy human donors (Canadian Blood Services Research Ethics Board Committee Protocol Reference #2005-003) by the Ficoll gradient method, according to manufacturer’s instructions (GE healthcare, Buckinghamshire, UK). Informed written consent was obtained from all subjects. CD4+ T-cells were then isolated from PBMCs using the EasySep negative selection human CD4+ T-cell enrichment kit, following the protocol by StemCell Technologies (Vancouver, Canada). For all experiments, isolated CD4+ T-cells were grown in complete RPMI-1640 media (Sigma-Aldrich, Oakville, Canada) containing 10% fetal bovine serum, 1% penicillin, 1% streptomycin and 0.1% gentamycin. Human embryonic kidney cells (HEK 293T, ATCC) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Burlington, Canada) with the same proportion of FBS and antibiotics as above. Cell cultures were incubated at 37°C in 5% CO2 atmosphere. CD4+ T-cells were activated by the addition of the following: 10 μg/mL anti-CD3 (UCHT1, Antibody Core Facility, Sunnybrook Research Institute, Toronto, Canada), 2 μg/mL anti-CD28 (10R-CD28bHU, Fitzgerald Industries International, Acton, USA) and 10 units/mL recombinant human Interleukin-2 (IL-2, Sigma-Aldrich).

**Virus Production:** We produced recombinant, replication deficient JR-FL enveloped (R5) or HXB2 enveloped (X4) HIV-1 containing a luciferase gene, as described previously [555]. Briefly, 2.5 x 10^6 HEK 293T cells were plated with 10 mL of DMEM 24 hours prior to transfection. We co-transfected 10 μg of HIV-1 NL4-3luc (luciferase gene inserted into viral nef gene and env deficient; a kind gift from Dr. Veneet KewalRamani, New York Medical University, NY) with 10 μg of JR-FL env plasmid (encodes the R5-tropic JR-FL glycoproteins; a generous gift from Dr. M. Tremblay, Quebec City, PQ) into each 10 cm plate of HEK 293T cells with the CalPhos™ Mammalian Transfection Kit (Clontech, Mountain View, USA), following the manufacturer’s instructions. This occurred in a biosafety level 3 (BSL3) lab. The same was performed for the HXB2 plasmid (HIV-1 NL4-3luc lacking nef and containing HXB2 (X4) env; another generous gift from Dr. M. Tremblay) at 15 μg per plate. DMEM media was replaced
after 24 hours and viral supernatant was collected on days 2 and 3. The supernatant was syringed through 0.45 μm pore filters (Millipore, Billerica, USA), then concentrated by underlaying with a 20% sucrose gradient and centrifuging at 16,000 x g for 90 minutes at 4 °C. Virus pellet were resuspended in TNE buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA and 100 mM NaCl) and stored in -80°C aliquots. p24 levels were measured by ELISA (ZeptoMetrix, Buffalo, USA). Replication competent IIIB (formerly HTLV-III B/H9), Ba-L (Reference ID: 85US_Ba-L) and KNH1207 (Reference ID: 00KE_KNH1207) viruses were acquired from the NIH AIDS Research & Reference Reagent Program’s International panel of 60 HIV-1 isolates (Germantown, USA). All viruses underwent one freeze-thaw cycle prior to infection studies.

**HIV-1 Infection and Luciferase Activity:** 2 x 10^5 CD4^+ T-cells pretreated with siRNA or drugs were infected with 1.0 ng of HXB2, 26 ng of JR-FL, 1.1 ng IIIB, 43 ng of Ba-L or 16 ng of KNH1207 at 37°C in 200 μL of clear growth media including activators in 96-well plates for 2 days. Cells were counted, while others were lysed with the Luciferase Assay System (Promega, Madison, USA). Other CD4^+ T-cells were infected with replication competent IIIB (X4), Ba-L (R5) or KNH1207 (R5), and integrated copies of provirus were quantified by qPCR after 12 hrs of infection.

**HIV-1 p24 ELISA:** 2 x 10^5 CD4^+ T-cells pretreated with siRNA were infected with IIIB or Ba-L for 1 hr at 37°C, washed in PBS three times, and incubated in 200 μL complete RPMI media supplemented with activators. Cell-free supernatant (180 μL) was collected on day 3 and assayed for p24^gag^ antigen following the manufacturer’s instructions (ZeptoMetrix, Franklin, USA).

**Drug Experiments:** Enriched CD4^+ T-cells were administered potent small molecule c-SRC inhibitors. Cells were given complete media containing activators, and one of the four inhibitors: dasatinib (BMS-354825), saracatinib (AZD0530), KX2-391 (Selleckchem, Houston, USA) or SRC Inhibitor-1 (Sigma-Aldrich). DMSO solvent served as a negative control. Twenty-four hours thereafter, cells were prepared for HIV-1 infection or FACS viability staining (Annexin V-FITC Apoptosis Detection Kit I, BD Pharmingen, Mississauga, Canada) to measure apoptosis and/or necrosis. In other experiments, enriched CD4^+ T-cells were given 20 μM of raltegravir 24 hours prior to HIV-1 infection.
**Flow Cytometry and Data Acquisition:** All antibodies were purchased from BD Pharmingen. Cell surface expression of CD3 and CD4 were determined on $1 \times 10^6$ CD4$^+$ T-cells after using the EasySep negative selection human CD4$^+$ T-cell enrichment kit (StemCell). In other experiments, cell surface expression of CD4, CXCR4 and CCR5 were determined on $2 \times 10^5$ enriched CD4$^+$ T-cells per tube, 48 hours after siRNA knockdown. In brief, cells were stained with 5 μL of PE mouse anti-human CD3, 5 μL of APC mouse anti-human CD4, 5 μL of FITC mouse anti-human CD4, 5 μL of PE mouse anti-human CXCR4, or 10 μL of Alexa Fluor®647 rat anti-human CCR5 in a 100 μL of FACS buffer (PBS + 2.5% FBS). Isotype control antibodies were FITC-conjugated mouse IgG1κ, PE-conjugated mouse IgG1κ or IgG2aκ, APC-conjugated mouse IgG1κ and Alexa Fluor®647-conjugated rat IgG2a. Data on 20,000 events per sample was collected using the Becton Dickenson FACSCalibur flow cytometer (BD Biosciences, Mississauga, Canada).

**Antibodies and Western Blot:** Activated CD4$^+$ T-cells ($1 \times 10^6$) infected with HXB2 or JR-FL virus for 1 hr were washed in PBS and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM HEPES, pH 7.3, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM Na$_3$VO$_4$, 50 M ZnCl$_2$, 2 mM EDTA and 2 mM PMSF). In other co-immunoprecipitation experiments, $3 \times 10^6$ activated CD4$^+$ T-cells were infected with HXB2 or JR-FL for 1 hr, lysed, and rotated at 4°C overnight with protein A/G PLUS-Agarose (Santa Cruz, Dallas, USA) and primary anti-v-SRC clone 327 (Millipore). After boiling samples, proteins from cell lysates were resolved on an 8%, reducing SDS-PAGE gel, and transferred to an Immobilon-P membrane for Western Blot detection. The blots were blocked with 1x Tris-buffer saline (TBS), 5% skim-milk powder and 0.5% Nonidet P-40 (BioShop, Burlington, Canada). Primary antibodies were diluted in 5% milk and 0.5% Nonidet P-40. Anti-GAPDH (Millipore), anti-PTK2B (BD Transduction Laboratories, Mississauga, Canada) and anti-c-SRC GD11 (Millipore) were used to probe the blots. To measure phospho-c-SRC (Tyr419, BD Transduction Laboratories) or phospho-PTK2B (Tyr402, New England Biolabs, Whitby, Canada), blots were stripped and then blocked with 1x TBS, 5% w/v BSA and 0.1% Tween-20. After washing, secondary goat anti-mouse HRP (Bio-Rad, Mississauga, Canada) or goat anti-rabbit HRP (Bio-Rad) antibody was added, followed by enhanced chemiluminescent (ECL) detection (GE
Healthcare). PTK2B and c-SRC protein levels were determined by comparing their densitometry to normalizer GAPDH, with the Molecular Imager GelDoc XR+ Imagin System (Bio-Rad).

**siRNA Knockdown Experiments:** Pools of four siRNA duplexes, specific for human c-SRC (cat # M-003175-03) or PTK2B (cat # M-003164-02) mRNA, and a control pool of non-targeting siRNA (cat # D-001206-13-05) were ordered from Dharmacon RNAi technologies (siGENOME SMART Pool, Thermo Scientific, Lafayette, USA). With enriched CD4⁺ T-cells, siRNA knockdown was performed using the Amaxa® Human T-cell Nucleofector® Kit (Lonza, Cologne, Germany), following the manufacturer’s instructions. Enriched T-cells (1.0 x 10⁷) were resuspended with 100 μL of Nucleofector solution, 900 nM of siRNA and 2 μL of siGuard RNase inhibitor® (Genlantis, San Diego, USA), then electroporated with program V-024. Cells were grown in 2 mL media lacking activators in a 37°C incubator for 12 hr, spun down, and resuspended in 2 mL complete media containing activators. Forty-eight hours post-transfection, cells were prepared for HIV-1 infection, harvested to measure protein knockdown of either c-SRC or PTK2B by Western blot densitometry, or prepared for FACS staining.

**qPCR Amplification of HIV-1 cDNA:** Genomic and viral cDNA was isolated from 5 x 10⁵ CD4⁺ T-cells, pre-treated by siRNA transfection and infected with HXB2 or JR-FL virus for 1 or 12 hours, using the QIAamp DNA Blood Mini Kit (Qiagen, Toronto, Canada). Samples were heated at 56°C for 2 hours in QIAamp DNA Blood Mini Kit buffer AL to deactivate viable viral particles.

The primer sets used to detect human or viral DNA (synthesized by the Center for Applied Genomics, The Hospital for Sick Children, Toronto, Canada) were as follows: β-globin forward, 5’-CCCTTGGACCAGGTTTCT -3’; β-globin reverse, 5’-CGAGCAGTTTCT TGCCATGAA-3’; early reverse transcripts (early RT) forward, 5’-GTAACTAGATTCCTCAG ACCCTTTTCT-3’; early RT reverse, 5’-TAGCAGTGGCCGCCGA-3’; late reverse transcripts (late RT) forward, 5’-CCGTCTGTTGTGTGACTCTGG-3’; late RT reverse, 5’-GAGTCCCTGCG TCGAGAGATCTG-3’; 2-LTR circles forward, 5’-CCCTCACCCCTTTTAGCAGTCAG-3’; 2-LTR circles reverse, 5’-TGGTGTGTCTTGCCAAATCA-3’; genomic Alu forward, 5’-GCCT CCCAAAG TGCTGGGATTACAG-3’; HIV-1 gag reverse, 5’-GCTCTGCGACCCCATCTCTCTCT
CC-3′; HIV-1 LTR forward, 5′-GCCTCA ATAAAGCTTGCCTTGA-3′; HIV-1 LTR reverse, 5′-TCCACACT GACTAAAAGGTCTGA-3′ [159, 542, 543]. For each qPCR run, 50-100 ng template DNA (with the exception of the no-template control, NTC) was added to PCR tubes containing: 12.5 μL 2x SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 300-900 nM of each forward and reverse primers, and PCR grade H2O (Roche Diagnostics, Indianapolis, USA) up to a final volume of 25 μL. PCR cycling parameters were as follows: initial denaturation at 95°C for 10 min; 40-50 cycles of amplification of 95°C for 15 sec, 58°C for 30 sec (β-globin or late RT, 54°C for early RT or 2-LTR) and 60°C for 30 sec. To measure integrated virus by Alu-gag PCR [542], tubes were first pre-amplified with 100 nM Alu forward and 600 nM gag reverse primers, with 2x SYBR Green PCR Master Mix as above, for 10 cycles of 93°C for 30 sec, 50°C for 60 sec, and 60°C for 100 sec. Nested PCR was performed on these samples by adding 300 nM each of HIV-1 LTR forward and reverse primers, with 2x SYBR Green PCR Master Mix, for 40 more cycles using the above parameters. Duplicate reactions were analyzed using the Rotor-Gene RG-3000 thermocycler (Corbett Research, Montreal, Canada). Five biological replicates in the c-SRC and PTK2B siRNA-treated groups were compared to the non-targeting siRNA-treated group by the 2^ΔΔCT comparative Ct method [544]. Absolute quantification was performed for PCR amplicons of integrated virus, relative to the chronically infected 8E5 cell line carrying one integrated proviral copy (NIH AIDS Reagent Program, Dr. Thomas Folks).

**In-Vitro Reverse Transcriptase Assay:** Quantification of reverse transcriptase activity from infected cell lysates was performed using the SYBR Green I qPCR-based product-enhanced RT (PERT) assay [556]. Briefly, 5 x 10^5 CD4^+ T-cells were infected with HXB2 or JR-FL virus for 1 hr, washed twice with PBS, lysed, diluted 10-fold with 10x sample dilution buffer, and given 1 μg of MS2 RNA template (Roche Diagnostics) to reverse transcribe in vitro. The cDNA product was measured by qPCR, and a standard curve of recombinant HIV-1 RT (Calbiochem) was used to assign enzyme activity units to reverse transcriptase from the infected cell lysate samples.

**Statistical Analysis:** Means were compared using a two-tailed, unpaired Student’s t test and corrected for multiple comparisons when more than two means were considered in an experiment, to minimize the probability of type 1 errors. FACS data shows the percent positive
cells collected with CellQuest software (BD Biosciences) and analyzed by FlowJo version X software. For all figures, an asterisk (*) denotes a \( p \) value < 0.05, two asterisks (**) denotes a \( p \) value < 0.01 and three asterisks (***) denotes a \( p \) value < 0.001. Error bars shown are the standard error around the mean (SEM).
3.4 Results

Kinase inhibitors targeting c-SRC significantly reduce HXB2 or JR-FL infection in activated CD4+ T-lymphocytes

To confirm and extend the finding that dasatinib inhibits HIV-1 production [554], we investigated whether pharmacological inhibitors of the SRC-family kinases, approved or under clinical investigation for the treatment of various cancers, could inhibit early HIV-1 infection of X4 or R5 luciferase-reporter viruses. For this purpose we tested dasatinib, saracatinib, a SRC/ABL ATP reversible inhibitor in a phase II clinical trial for the treatment of advanced ovarian cancer (http://clinicaltrials.gov/ct2/show/NCT00610714); KX2-391, a SRC substrate competitive inhibitor in a phase II clinical trial evaluating efficacy in gastric and breast cancers (http://clinicaltrials.gov/ct2/show/NCT01764087); and SRC Inhibitor-1, a dual ATP and substrate competitive inhibitor in pre-clinical studies [557].

We first isolated peripheral blood mononuclear cells (PBMCs) from the whole blood of healthy human donors, and could enrich a CD3+CD4+ cell population to 98% purity with a negative selection human CD4+ T-cell enrichment kit (Fig. 3.1a). No increase in apoptosis or necrosis was observed after treating activated CD4+ T-cells with the four SRC inhibitors at the concentrations tested (Fig. 3.2). Following drug-treatment and activation of primary CD4+ T-cells for 24 hrs, we measured a strong decrease in luciferase activity after two days of HXB2 (X4) or JR-FL (R5) viral infection (Fig. 3.3). The dasatinib treatment exhibited the greatest potency at doses in the 10-100 nM range.
**Fig. 3.1: Cell Purity and Cell Viability of Enriched CD4⁺ T-Cells nucleofected with siRNA.**

PBMCs were isolated from a healthy human donor by the Ficoll gradient method. CD4⁺ T-cells were then enriched using the EasySep negative selection human CD4⁺ T-cell enrichment kit. (a) One million cells were prepared for FACS and stained with Propidium Iodide (PI), CD4-APC and CD3-PE. Cells were gated on the PI negative population. This FACS plot is representative of three independent experiments. (b) Cell viability was assessed by 7-AAD staining 48 hrs post-siRNA knockdown, on untreated CD4⁺ cells (un.), electroporated cells (mock), and cells transfected with siRNA. Displayed is the average of two independent experiments, with the error bars showing the range.
Fig. 3.2: SRC-Family Drug Inhibitors do not Increase Necrosis or Apoptosis of Activated CD4+ T-Lymphocytes. Enriched CD4+ T-cells isolated from a healthy donor were given one of four SRC-family inhibitors (dasatinib, saracatinib, KX2-391, SRC Inhibitor-1) or DMSO solvent (-ve control), and activated in growth media for 24 hrs. One million cells were then prepared for FACS and stained with Propidium Iodide and Annexin V-FITC. No increase in necrosis/apoptosis was observed for most drug treatments, relative to DMSO control. Ten μM of KX2-391 or SRC Inhibitor-1 showed a slight increase in both PI and Annexin V stained cells, suggesting increased late apoptotic cells.
Fig. 3.3: Pre-treating CD4⁺ T-cells with c-SRC kinase inhibitors prior to HXB2 or JR-FL infection. Enriched CD4⁺ T-cells were administered DMSO solvent (-ve control) or one of four SRC-family kinase inhibitors (dasatinib, saracatinib, KX2-391 or SRC Inhibitor-1), and grown in growth media containing activators for 24 hrs. Cells (2 x 10⁵) in 200 μL clear media were infected with nef deficient, luciferase reporter viruses: 1.0 ng of HXB2 (a) or 26 ng of JR-FL (b). After 48 hrs, cells were lysed and luciferase activity was detected. The means of all drug treatments were compared with the mean of the DMSO solvent treatment. Five biological replicates were run in each treatment. Error bars are standard error of the mean.
c-SRC and PTK2B become activated and co-immunoprecipitate shortly after HXB2 or JR-FL infection

Given the SRC-family kinase inhibitor findings, we asked whether c-SRC signaling via PTK2B plays an important role during early viral entry. c-SRC protein expression was strongly induced in CD4+ T-cells 48 hrs after stimulating with anti-CD28, anti-CD3 and IL-2 (Fig. 3.4a). PTK2B expression remained unaffected during T-cell activation. Meanwhile c-SRC became phosphorylated at Tyr419 1 hr following HXB2 (X4) or JR-FL (R5) infection, a key phosphorylation step that leads towards high c-SRC activity [558]. PTK2B also became phosphorylated at Tyr402 after 1 hr of infection, suggesting SRC-family phosphorylation of PTK2B [559]. Immunoprecipitation of c-SRC followed by probing for phosphorylated PTK2B revealed the two kinases strongly co-associate after 1 hr of HXB2 or JR-FL infection, and that PTK2B has been cleaved into a smaller fragment (Fig. 3.4b).
Fig. 3.4: c-SRC and PTK2B activation shortly after HIV-1 infection. (a), Western blot of protein expression and phosphorylation state of c-SRC and PTK2B from the cell lysate of 1 million enriched CD4⁺ T-cells. Purified recombinant human pp60c-SRC protein was loaded in lane 1. Cell lysates were isolated from PBMCs of a healthy donor (lane 2), activated for 48 hrs (2 μg/mL CD28bHu, 10 μg/mL UCHT-1 and 10 units/mL recombinant IL-2, lane 3) and infected with HXB2 or JR-FL virus for 1 hr (lanes 4 and 5). (b), Immunoprecipitation of c-SRC protein from CD4⁺ T-cell lysate, followed by probing the Western blot with anti-Y402 PTK2B.
c-SRC and PTK2B siRNA knockdown reduce the infection of luciferase reporter viruses and replication competent viruses

To test whether siRNA knockdown of either kinase reduces viral infection, we electroporated siRNA into primary CD4\(^+\) T-cells, performed activation, and measured protein knockdown 48 hrs thereafter (Fig. 3.5a-b). We could consistently reduce c-SRC expression by 40% and PTK2B expression by 80%. Cell viability at this time-point ranged from 70-80% (Fig. 3.1b). We then infected the siRNA-transfected, activated CD4\(^+\) T-cells with HXB2 virus, and observed decreased luciferase activity in c-SRC siRNA-treated, but not in PTK2B siRNA-treated cells, relative to the non-targeting siRNA control (Fig. 3.5c). In contrast, siRNA knockdown of either c-SRC or PTK2B reduced the luciferase activity following JR-FL viral infection (Fig. 3.5d). This suggests that the co-receptor engaged upon entry (CXCR4 or CCR5) affects downstream signaling events, with c-SRC common to the entry of both viruses tested and PTK2B playing a role primarily during JR-FL (R5) infection. Further supporting this evidence, we also found that knockdown of only c-SRC reduced viral integration of replication competent X4 IIIB, while knockdown of either kinase reduced integration of R5 viruses Ba-L and clinical isolate KNH1207 (Fig. 3.5e). Indeed, the unique response of X4 and R5 viruses to c-SRC or PTK2B siRNA also affected the later stages of the replication cycle of IIIB or Ba-L, as determined by p24 detected in cell-free supernatant three days post-infection (Fig. 3.5f-g).
Fig. 3.5: Luciferase reporter activity, viral integration and p24 production after c-SRC or PTK2B siRNA knockdown. (a), c-SRC and PTK2B protein knockdown 48 hrs after electroporating $1.0 \times 10^7$ enriched CD4$^+$ T-cells with 900 nM of non-targeting (NT), c-SRC- or PTK2B-targeting siRNA. (b), Densitometry of protein knockdown relative to GAPDH from panel A. (c and d), Forty-eight hours post-siRNA nucleofection and T-cell activation, $2 \times 10^5$ enriched CD4$^+$ T-cells were infected with 1.0 ng of HXB2 or 26 ng of JR-FL. Luciferase activity was measured from cell lysates two days thereafter. (e), In separate experiments, $2 \times 10^5$ enriched CD4$^+$ T-cells were infected with replication competent X4 IIIB (1.1 ng), R5 Ba-L (43 ng) or R5 clinical isolate KNH1207 (16 ng). Absolute copy number of integrated provirus was measured 12 hrs post-infection by qPCR. (f and g), p24 detection three days after IIIB or Ba-L infection in cells nucleofected with siRNA. The means shown are of five biological replicates. Error bars are standard error of the mean.
siRNA knockdown of c-SRC or PTK2B did not reduce surface expression of receptors required for viral entry or reverse transcriptase activity

We then determined whether c-SRC or PTK2B siRNA knockdown altered the surface expression of CD4 or the chemokine co-receptors, CXCR4 or CCR5, which are necessary for HIV-1 binding and fusion. Surface expression of CD4, CXCR4 and CCR5 was not affected by siRNA knockdown of either kinase (Fig. 3.6a-b). We then infected the siRNA-transfected, activated CD4⁺ T-cells with HXB2 or JR-FL virus. After measuring reverse transcriptase (RT) activity 1 hr post-viral infection, we found no significant changes in RT activity following c-SRC or PTK2B siRNA knockdown, relative to the non-targeting siRNA control (Fig. 3.6c-d).
Fig. 3.6: **Cell surface receptor expression and RT activity.** (a and b), T-cell surface expression of CD4, CXCR4 and CCR5 48 hrs post-siRNA nucleofection, gating on 7-AAD− cells. (c and d), 5 x 10^5 siRNA-treated and activated CD4^+ T-cells were infected with 1.0 ng of HXB2 or 26 ng of JR-FL in 200 μL of growth media containing activators. Cell lysates were collected after 1 hr to measure *in vitro* reverse transcriptase activity. The means shown are of five biological replicates. Error bars are standard error of the mean.
Suppressing c-SRC or PTK2B with siRNA reduced viral integration and increased late reverse transcripts and 2-LTR circles

Next we explored at which stage following reverse transcription c-SRC and PTK2B are exerting their effects, by measuring viral synthesis of early reverse transcripts (Early RT), late reverse transcripts (Late RT), integrated virus in the host genome, and two-long terminal repeat (2-LTR) circular DNA, which are indicative of failed integration [56]. Primer efficiency curves, time-course experiments to optimally measure viral cDNA, and qPCR product melting curves are presented in Figs. 3.7 and 3.8. We observed a 4-fold reduction in integration of either HXB2 or JR-FL viruses in the c-SRC siRNA-treated cells (Fig. 3.9a-b). Surprisingly, this was associated with increased early reverse transcripts, late reverse transcripts, as well as 2-LTR circles. These experiments are the average of three separate donors (see Fig. 3.10 for the three independent experiments). PTK2B siRNA knockdown also showed a similar trend as c-SRC siRNA knockdown during JR-FL infection, but not with HXB2 infection. This is consistent with the luciferase findings in Fig. 3.5c-d. The qPCR data suggest multiple interactions of both kinases during HIV-1 entry, as we observed decreased integration that coincides with increased early transcripts, late reverse transcripts and 2-LTR circles. The increase in late reverse transcripts caused by c-SRC or PTK2B siRNA knockdown was not due to increased reverse transcription (Fig. 3.6c-d), but rather, taken together with the observed increase in 2-LTR circles and decrease in integrated provirus, that both kinases likely have a role in pre-integration complex (PIC) formation or nuclear import of viral cDNA. Their nuclear affects on 2-LTR circles and integration are akin to the integrase inhibitor raltegravir in HXB2 or Ba-L infection (Fig. 3.9c). Interestingly, JR-FL virus appears resistant to high-dose raltegravir treatment, despite being susceptible to c-SRC or PTK2B siRNA knockdown and SRC-family kinase inhibitors (Fig. 3.1b).
Fig. 3.7: qPCR and PERT Assay Standard Curves. To satisfy the assumptions of the $2^{\Delta\Delta CT}$ comparative C_T method [544], primer efficiency curves were performed on serial dilutions of DNA from CD4⁺ T-cells infected with HXB2 virus. Primer sets were tested for $\beta$-globin (a), early reverse transcripts (b), late reverse transcripts (c), 2-LTR circles (d) and integrated virus (e). A standard curve of DNA extracted from 8E5 cells was also constructed to convert cycle threshold into copies of integrated virus (f). For the PERT assay, a standard curve of recombinant reverse transcriptase (RT) was used to assign enzyme activity units to reverse transcriptase in infected cell lysate samples (g). It was expected the efficiency curve of recombinant RT would be less than 1.00, as the RNAse inhibitor added to prevent MS2 RNA degradation in the PCR reaction is a known reverse transcriptase inhibitor [546]. Each data point is the mean of technical triplicate PCR reactions. Red data points are DNA concentrations beyond the linear range of qPCR detection.
**Fig. 3.8: Time-Course and qPCR Melting Curves of HIV-1 DNA Targets.** 5 x 10^5 CD4^+ T-cells were activated for 48 hrs and then infected with 1.0 ng of HXB2 or 26 ng of JR-FL in complete media containing activators. **A-D**, Early reverse transcripts (Early RT), late reverse transcripts (Late RT), 2-LTR circles and integrated HIV-1 were measured in DNA extracted from cell lysates 0.5-48 hrs post-infection. Time points in grey represent when each transcript was measured in subsequent experiments. **E-H**, Representative melting curves of qPCR products (blue) relative to no template control reactions (NTC, black) demonstrate the specificity of the published primer sets. The heterogeneous mixture of Alu-gag amplified sequences during the exponential phase explains the various melting peaks seen after the kinetic phase of nested PCR in panel H.
Fig. 3.9: qPCR of early HXB2 or JR-FL infection after c-SRC or PTK2B siRNA knockdown. (a and b), Forty-eight hours post-siRNA nucleofection and T-cell activation, $5 \times 10^5$ enriched CD4$^+$ T-cells were infected with 1.0 ng of HXB2 or 26 ng of JR-FL. Cells were lysed after 12 hrs of infection to measure early reverse transcripts (Early RT), late reverse transcripts (Late RT), 2-LTR circle formation or integrated virus. qPCR of HXB2 or JR-FL DNA was normalized to genomic $\beta$-globin, and graphed relative to the non-targeting (NT) siRNA treatment (x-axis). The means shown are the average of three different donors from three independent experiments (Fig. 3.10 shows these separately). (c), Enriched CD4$^+$ T-cells ($2 \times 10^5$) were incubated with 20 μM raltegravir 24 hours prior to infection with HXB2, Ba-L or JR-FL. qPCR products of HIV-1 were measured as in panels a and b. Error bars are standard error of the mean.
Fig. 3.10: qPCR of HXB2 or JR-FL Infection in CD4+ T-Cells from Three Separate Donors. Three independent experiments of HXB2 or JR-FL infection measured by qPCR. CD4+ T-cells from three donors were isolated, and were electroporated with 900 nM of non-targeting (NT), c-SRC or PTK2B siRNA. Cells were activated in complete media 12 hrs later, and at 48 hrs post-transfection, 5 x 10^5 T-cells were infected with 1.0 ng of HXB2 (a-c) or 26 ng of JR-FL (d-f). Early reverse transcripts (E. RT) and late reverse transcripts (L. RT) were measured after 1 hr of infection, while 2-LTR circles (2-LTR) and integration (Int. HIV-1) were quantified from cell lysates collected after 12 hrs of infection. All qPCR targets were normalized to genomic β-globin, and cells treated with NT siRNA were the reference treatment set to 1-fold (x-axis). The means of c-SRC or PTK2B siRNA treatments were compared with the NT siRNA treatment, for a given HIV-1 qPCR target. Technical duplicates were performed, and means are the average of five biological replicates. Error bars are standard error of the mean.
3.5 Discussion

Efficacious and well-tolerated therapeutics that reduce virological and immunological failure are key to designing new HIV-1 regimens. Our study demonstrates a new means to inhibit HIV-1 integration and modulate early HIV-1 entry in CD4\(^+\) T-lymphocytes by targeting specific host non-receptor tyrosine kinases (summary schematic Fig. 3.11). Our work herein is the first report to show a specific role for the protein tyrosine kinase, c-SRC, in facilitating HIV-1 infection of X4 and R5 viruses.

Fig. 3.11: Summary schematic of the proposed mechanism for c-SRC involvement during early HIV-1 infection.
We first demonstrate that four SRC-family kinase inhibitors reduced HXB2 or JR-FL infection by as much as 90% in activated CD4+ T-cells (Fig. 3.3). The diversity of mechanisms by which these four drugs inhibit c-SRC, and differential on- and off-target efficacy, provide a strong rationale for testing these inhibitors for their ability to reduce in vivo HIV-1 infection in a humanized mouse model. While preclinical-stage inhibitors targeting other host kinases have been found to influence viral infection (c-Jun N-terminal Kinase (JNK) inhibitors that reduce integrase stability, protein kinase C agonists that reactivate latent HIV-1 provirus to purge latent reservoirs [560]), our study is the first to describe mechanisms by which clinically approved (in the case of dasatinib), safe, and well-tolerated SRC-family inhibitors act on early HIV-1 infection, suggesting these inhibitors could be promptly repurposed for potential treatment of HIV-1 infection. For instance, a 75 nM dose of dasatinib has recently been shown to strongly inhibit p24 production in activated CD4+ T-cells cultured from HIV+, treatment-naïve patients [554]. This dasatinib concentration was below the well-tolerated in vivo level of 180 nM [561], and Pogliaghi et al. reported no increase in cell toxicity in cultured CD4+ T-cells [554]. Moreover, dasatinib has recently been shown to synergistically enhance the antiviral properties of sofosbuvir, further inhibiting in vitro hepatitis C infection [532]. It also has potency in inhibiting dengue virus 2 RNA replication by inhibiting the SRC-family kinase member FYN [178].

To investigate potential targets of these four SRC kinase inhibitors, we demonstrated c-SRC and its binding partner PTK2B become activated within 1 hr of HXB2 or JR-FL infection, and that phosphorylated PTK2B co-immunoprecipitates with c-SRC after infection (Fig. 3.4). Activity of calpains, which are Ca2+-dependent proteases known to cleave PTK2B at focal adhesions [336], explains why a smaller PTK2B fragment immunoprecipitated with c-SRC in Fig. 3.4B. The activation or autophosphorylation of cleaved PTK2B at tyrosine 402 may be induced in response to chemokine engagement [147] or activation of the purinergic receptor P2Y2 during early HIV-1 infection [345]. This may form a signaling complex that contains c-SRC and actin interacting proteins that facilitate early HIV-1 entry and subsequent formation of the reverse transcriptase complex in the cytosol. We then showed that reducing c-SRC protein with targeted-siRNA reduced the luciferase activity of HXB2 and JR-FL viruses, and decreased the copy number of
integrated IIIB, Ba-L or KNH1207 provirus (Fig. 3.5). *c-SRC* siRNA knockdown affects HIV-1 infection at a stage following reverse transcription and prior to genomic integration (Fig. 3.6 and 3.9). We also show a similar finding with *PTK2B* siRNA knockdown, reducing JR-FL luciferase reporter activity and viral integration (Fig. 3.5d and 3.9b). However, *PTK2B* siRNA knockdown had little effect on the entry of the X4 viruses HXB2 or IIIB (Fig. 3.5c, 3.5e and 3.9a). While PTK2B activation has been reported to be downstream of both CXCR4 and CCR5 co-receptor engagement with gp120/gp41 in a T-cell line [147], our finding suggests that PTK2B signaling is more dispensable during HXB2 (X4) infection in primary CD4+ T-cells. It is possible that engagement of CXCR4, abundantly expressed on activated CD4+ T-cells compared to CCR5 (Fig. 3.6a-b), may provide a more robust signal that causes increased activation of c-SRC independently of PTK2B activation.

It is intriguing that reducing either kinase had no effect on JR-FL reverse transcription (Fig. 3.6d) yet inhibited viral integration (Fig. 3.9b). Meanwhile, the same siRNA knockdown conditions increased reverse transcription and viral integration in our previous report on transformed T-cell lines [555]. Jurkat T-cells show constitutive c-SRC and PTK2B expression and activation, as well as other signaling abnormalities downstream of the T-cell receptor [562], suggesting a very different intracellular environment compared with activated primary CD4+ T-cells. Indeed, our work is adding to a growing body of evidence that the involvement of receptor and non-receptor tyrosine kinases during HIV-1 infection is highly dependent on the cell model investigated [289, 563, 564], and that transformed cell lines may not be physiologically relevant models of signal transduction during *in vivo* HIV-1 infection.

Our findings also suggest c-SRC signaling is a common feature of the early HIV-1 life cycle, and that its interaction with viral and host proteins during PIC formation, viral cDNA nuclear transport and genomic integration warrants further research. In light of the 20-fold increase in late reverse transcripts following *c-SRC* siRNA knockdown (Fig. 3.9a), our findings offer the potential to advance the study of host proteins integral to the PIC. Cellular kinases have been reported to directly phosphorylate viral PIC proteins (reviewed in [155]) and assist with integration into the host genome [154, 159]. Moreover, the identities of tyrosine kinases that phosphorylate known viral constituents of the PIC remain unknown. For instance, the tyrosine
kinases that phosphorylate matrix protein p17 at C-terminal Y132 and preferentially target matrix protein to the nucleus, have yet to be identified [158]. Future experiments using knockdown of c-SRC or PTK2B could reveal whether these tyrosine kinases directly phosphorylate host or viral PIC proteins, and could also uncover novel protein interactions for pharmacological intervention. Whether nuclear c-SRC directly participates in HIV-1 integration warrants further research. Moreover, c-SRC siRNA knockdown produced a viral qPCR signal reminiscent of integrase inhibitors (Fig. 3.9c): reduced viral integration and increased 2-LTR circles [565-567]. siRNA targeting PTK2B has also been shown to inhibit mutated HIV-1 strains that are resistant to reverse transcriptase or integrase inhibitors [345]. The unexpected finding that JR-FL was resistant to raltegravir treatment opens up the possibility of c-SRC or PTK2B inhibitors substituting for integrase inhibitors when combination therapies fail due to integrase mutations. This warrants further experiments in humanized animal models to explore how these drugs can improve existing HIV-1 treatment regimens.

In conclusion, our findings demonstrate the importance of host non-receptor tyrosine kinases in the early stages of HIV-1 entry, specifically c-SRC and to a lesser extent PTK2B. Furthermore, we demonstrate that these kinases can have multifunctional roles when interacting with the virus at various stages of viral entry. The mechanism by which reducing or inhibiting c-SRC or PTK2B restricts viral integration remains to be explored. Nonetheless, the availability of well-studied SRC-family kinase inhibitors of known efficacy, specificity, pharmacokinetics and toxicity profile, opens up new avenues for repurposing these drugs in combination with known antivirals as novel means to inhibit HIV-1 and other viral infections. In particular, these may prove useful for patients with contraindications for current HIV-1 drug combinations.
Role of author McCarthy S.D.: Illustrated Figure 4.1, and performed all experiments for Figures 4.2, 4.3 and Figures 4.5-4.8. I measured % inhibition for the majority of Figure 4.4. I also performed all experiments for Tables 4.1 and 4.2. I analyzed all the data, co-wrote the manuscript draft, and performed experiments to address reviewer’s comments.

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4.1 Abstract

To date there are no approved antiviral drugs for the treatment of Ebola virus disease (EVD). While a number of candidate drugs have shown limited efficacy in vitro and/or in non-human primate studies, differences in experimental methodologies make it difficult to compare their therapeutic effectiveness. Using an in vitro model of *Ebola Zaire* replication with transcription-competent virus like particles (trVLPs), requiring only level 2 biosafety containment, we compared the activities of the type I interferons (IFNs) IFN-α and IFN-β, a panel of viral polymerase inhibitors (lamivudine (3TC), zidovudine (AZT) tenofovir (TFV), favipiravir (FPV), the active metabolite of brincidofovir, cidofovir (CDV)), and the estrogen receptor modulator, toremifene (TOR), in inhibiting viral replication in dose-response and time course studies. We also tested 28 two- and 56 three-drug combinations against Ebola replication. IFN-α and IFN-β inhibited viral replication 24 hours post-infection (IC$_{50}$ 0.038 µM and 0.016 µM, respectively). 3TC, AZT and TFV inhibited Ebola replication when used alone (50-62%) or in combination (87%). They exhibited lower IC$_{50}$ (0.98-6.2 µM) compared with FPV (36.8 µM), when administered 24 hours post-infection. Unexpectedly, CDV had a narrow therapeutic window (6.25-25 µM). When dosed > 50 µM, CDV treatment enhanced viral infection. IFN-β exhibited strong synergy with 3TC (97.3% inhibition) or in triple combination with 3TC and AZT (95.8% inhibition). This study demonstrates that IFNs and viral polymerase inhibitors may have utility in EVD. We identified several 2 and 3 drug combinations with strong anti-Ebola activity, confirmed in studies using fully infectious ZEBOV, providing a rationale for testing combination therapies in animal models of lethal Ebola challenge. These studies open up new possibilities for novel therapeutic options, in particular combination therapies, which could prevent and treat Ebola infection and potentially reduce drug resistance.
4.2 Introduction

As of December 13, 2015, the current outbreak of Ebola virus disease (EVD) in West Africa resulted in 28,633 cumulative cases and 11,314 deaths [568]. Two potential vaccine candidates, rVSV∆G-ZEBOV and ChAd3-EBO Z, have shown durable protection from lethal Ebola challenge in mice [569] and macaques [32] respectively, and are part of the phase II/III PREVAIL trial in Liberia and Guinea (https://clinicaltrials.gov/ct2/show/NCT02344407). Other potential therapeutics, such as convalescent plasma and the antibody cocktail ZMapp [30] have been approved for an emergency phase II/III trial in Guinea (https://clinicaltrials.gov/ct2/show/NCT02342171) and a phase I trial in Liberia (https://clinicaltrials.gov/ct2/show/NCT02363322), respectively. However, to date there is no licensed vaccine or treatment for EVD, although improvements in supportive care are increasing survival rates [570].

Repurposing antivirals used for other viral infections, based on knowledge of mechanisms of action, has prompted accumulating interest in the application of different nucleoside/nucleotide analogues and type I interferons (IFNs) for the treatment of Ebola virus disease (EVD). Experimental nucleoside analogues may have therapeutic efficacy for EVD, given the evidence of protection in primate and rodent disease models, 2-6 days after lethal Ebola or the related hemorrhagic Marburg virus challenges [23, 418]. Favipiravir (FPV), a viral polymerase inhibitor, provides 100% protection when administered 6 days after challenge with a lethal dose of Ebola virus [23] and has been evaluated in the phase II/III JIKI trial in Guinea (https://clinicaltrials.gov/ct2/show/NCT02329054). TKM-Ebola, a cocktail of siRNAs targeting VP35 and L polymerase and brincidofovir (BCV), a viral polymerase inhibitor that has activity against dsDNA viruses such as adenovirus and cytomegalovirus [571], were also considered for treatment against EVD. The brincidofovir trial was halted, ostensibly because of projections of low recruitment.

Despite infecting different target cells, Ebola and HIV-1 share many similar features early in their replication cycle. Both are RNA viruses that package a viral polymerase (L for Ebola, RT for HIV-1) required for early replication in the cytosol of the host cell [572]. Homology-based
structural prediction of the RNA-dependent RNA polymerase of Ebola indicates the polymerase contains conserved structural motifs in the catalytic palm subdomain similar to viral DNA polymerases [455], supportive of nucleoside analogues potentially inhibiting Ebola replication. Inhibiting HIV-1 reverse transcription with nucleoside analogues such as lamivudine (3TC, cytidine analogue), zidovudine (AZT, thymidine analogue) or tenofovir (TFV, adenosine monophosphate analogue) is the basis for highly active antiretroviral treatment (HAART) [523, 573]. Nucleoside analogues are on the WHO list of essential medicines and can be deployed in limited resource settings [574]. Moreover, AZT binds RNA through G-C and A-U bases [575], prompting us to evaluate whether these nucleoside analogues might also inhibit Ebola replication.

Type I IFNs mediate diverse biological effects, including cell type-independent antiviral responses and cell type-restricted responses of immunological relevance. IFNs inhibit viral infection by preventing viral entry into target cells and by blocking different stages of the viral replication cycle for different viruses. Moreover, type I IFNs have a critical role in linking the innate and adaptive immune responses to viral challenge. IFN-α/β expression occurs as the earliest non-specific response to viral infection. Indeed, viruses have evolved immune evasion strategies specifically targeted against an IFN response, confirming the importance of IFNs as antivirals. This immune evasion strategy is relevant when one considers the IFN response to Ebola infection [576]. Ebola proteins VP24 and VP35 inhibit host cell systems that lead to IFN production and also inhibit events associated with an IFN response [577-579]. VP24 blocks the binding of importins to phosphorylated STAT1, preventing STAT1 nuclear translocation required for transcription of interferon simulated genes [577]. VP35 binds viral dsRNA, preventing dsRNA degradation [578] and inhibits the phosphorylation of IRF-3 and the SUMOylation of IRF-3 and IRF-7, thereby limiting IFN production [579]. Despite these virally-encoded mechanisms to limit an IFN response to infection, different rodent and non-human primate studies provide evidence for IFN-induced partial protection: the effects of IFN-α/β treatment in lethal Ebola virus infection reduced viremia and prolonged survival [31, 580, 581]. Thus, a potential therapeutic effect for IFNs as monotherapy in EVD, or in combination with other anti-Ebola therapies, has not been resolved.
4.3 Materials and Methods

**Cell culture and trVLP infection:** We employed an established mini-genome system to rapidly evaluate candidate drugs that could inhibit *Ebola Zaire* replication under BSL2 conditions (see Fig. 4.1 for an an illustration of the reporter system) [534]. The mini-genome encodes 3 of the 7 ZEBOV proteins (VP24, VP40 and GP1,2) and a luciferase reporter gene. Expression plasmids for the remaining four Ebola nucleocapsid proteins (L, NP, VP30 and VP35) were also included during transfection. Cell culture conditions and virus infections were performed as previously described [534]. Briefly, 80,000 producer 293T cells (American Type Culture Collection; ATCC, Rockville, USA) were seeded in individual wells of 24-well plates in 400 μL Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS, 1% penicillin and 1% streptomycin, and grown in 5% CO2 atmosphere at 37°C. Cells were transfected with the viral replication protein plasmids (L, NP, VP30, VP35), a tetracistronic Ebola mini-genome and the T7 polymerase, using the CalPhos Mammalian Transfection Kit (Clontech Laboratories). Twenty-four hours later, medium was replaced with 800 μL DMEM with 5% FBS. The replication and transcription-competent virus like particles (trVLPs) were harvested 3 days later. Virus stock was frozen at -80°C.

For infection, 293T target cells were seeded at 80,000 cells in 400 μL of DMEM supplemented with 10% FBS. Target cells were then transfected with the four viral replication protein plasmids, as well as Tim-1, to allow efficient virus binding and entry. Twenty-four hours post-transfection, 25 μL of trVLP stock was diluted in 600 μL of DMEM with 5% FBS, warmed to 37°C for 30 min, then added to target cells. Medium was removed the following day and replaced with 800 μL DMEM with 5% FBS. Four days post-infection, the medium was aspirated and cells were re-suspended in 200 μL of 1x *Renila* Luciferase Assay Lysis Buffer (*Renilla* Luciferase Assay System, Promega). Lysates were assayed for luciferase activity.

**ZEBOV-eGFP infection:** We generated recombinant ZEBOV expressing enhanced green fluorescent protein (eGFP) from cDNA clones of full-length infectious ZEBOV, as previously described [582]. The eGFP reporter protein was expressed as an eighth gene, and the virus exhibited an *in vitro* phenotype similar to wild type ZEBOV. Notably, *in vivo*, incorporation of
Fig. 4.1: Transcription and replication competent virus-like particle (trVLP) assay.

ZEBOV trVLP particles were collected from the supernatant of 293T producer cells. These virus particles carried tetracistronic RNA genomes that contain a renila luciferase reporter gene, VP40, GP$_{1,2}$ and VP24. Target 293T cells were transected with expression plasmids prior to infection: Tim-1 to allow efficient viral entry and RdRP L, NP, VP30 and VP35 to facilitate viral replication. Cytosolic expression of these four additional viral proteins allowed formation of the RNA-dependent RNA replication complex after receptor-mediated endocytosis of trVLP virus. Luciferase activity detected in cell lysates indicated successful viral transcription and translation of the ZEBOV mini-genome.
GFP into wild type ZEBOV results in some attenuation of disease [582]. All work with infectious ZEBOV was performed in biosafety level 4 (BSL4), at the National Microbiology Laboratory of the Public Health Agency of Canada in Winnipeg, Manitoba. 293T cells (30,000) were seeded in 96-well plates in 100 μL DMEM with 10% FBS. Twenty-four hours thereafter, the medium was replaced with 100 μL DMEM with 10% FBS containing ZEBOV-GFP at an MOI of 0.1. Twenty-four hours post-infection, the medium was removed and replaced with 200 μL of DMEM with 5% FBS, or 190 μL DMEM with 5% FBS and 10 μL of single or combinations of drugs. eGFP fluorescence was measured 3 days post-infection using a Synergy HTX Multi-Mode Microplate Reader (BioTek).

**Drugs:** For these experiments, we used toremifene citrate (TOR; Sigma), cidofovir hydrate (CDV; Sigma) favipiravir (FPV, T-705; Cellagen Technology), lamivudine (3TC; Sigma) zidovudine (AZT), tenofovir (TFV) maraviroc (MVC; NIH AIDS Reagent Program), Infergen (IFN alfacon-1, Pharmunion Bsv Development Ltd.) or human interferon beta-1a (IFN-β, Avonex; Biogen).

**Mini-genome RNA extraction and qRT-PCR quantification of viral RNA:** Forty-eight hours after trVLP infection, medium was aspirated from 293T cells that had either been left untreated or treated with the various drugs and total RNA extracted from cell lysates with 500 μL of TRIzol (Thermo Fisher Scientific). cDNA synthesis was performed on 5 μg of total RNA, using the First-Strand cDNA Synthesis Kit (GE Healthcare Life Sciences), according to the manufacturer’s instructions. A 20 μl reaction also contained bulk first-strand cDNA reaction mix, DTT solution and 40 pmol of one of two trVLP specific primers [583]: vRNA forward (5’-GGC CTC TTC TTA TTT ATG GCG A -3’), or cRNA/mRNA reverse (5’-AGA ACC ATT ACC AGA TTT GCC TGA-3’). Both primers were synthesized by the Center for Applied Genomics (The Hospital for Sick Children, Toronto, Canada). Real-time qPCR reactions (25 μl) were conducted in duplicate, using the Rotor-Gene RG-3000 thermocycler (Corbett Research, Montreal, Canada). Each reaction contained 100 ng template cDNA, 12.5 μL 2 x SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 300 nM of both the forward (vRNA) and reverse (cRNA/mRNA) primers, and PCR grade H₂O (Roche Diagnostics, Indianapolis, USA). Samples lacking reverse transcriptase (No RT) during first-strand cDNA synthesis served
as negative controls. Cycling parameters were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of amplification with 95°C for 15 seconds, 56°C for 30 seconds, and 60°C for 30 seconds. Biological triplicates in the drug-treated groups were normalized to the average Ct of infected cells given DMSO solvent alone, by the $2^{\Delta\text{CT}}$ comparative C_T method.

**Cell viability assay:** Dose-response cytotoxicity/viability assays were conducted in 293T cells 4 days post-infection for each of the drugs examined, either alone or in the various combinations indicated, using the MTT assay as previously described [584].

**Statistics:** Means were compared using a two-tailed, unpaired Student’s t test and corrected for multiple comparisons. For all figures, (*) denotes a p value <0.05, (**) denotes a p value <0.01 and (***) denotes a p value <0.001. Error bars shown are the standard error around the mean (SEM). Synergy between two and three-drug combinations and combination index (CI) were calculated with CompuSyn Version 1.0 [533]. The coefficient of determination ($R^2$) was determined for simple linear regressions.
4.4 Results

We employed an established mini-genome system to rapidly evaluate candidate drugs that could inhibit *Ebola Zaire* replication under BSL 2 conditions [534, 535, 585]. At the outset, we established the experimental conditions for infection with replication and transcription-competent virus like particles (trVLPs), by examining luciferase activity under various transfection and drug treatment conditions, which included transfection with viral support protein plasmids (Fig. 4.2). We included treatment with maraviroc, a CCR5 inhibitor, that would have no effect on trVLP entry and infection, thereby serving as a negative control for subsequent treatment regimens. The triple combination of 3TC + AZT + TFV significantly inhibited trVLP infection, and further inhibited luciferase activity of a second passage of 293T cells when administered supernatant of a previous passage of cells.

In a second series of experiments, we examined the inhibitory effects of IFN-α (0.5 µM/10,000 U/mL), IFN-β (0.2 µM/1,000 U/ml), TOR (5 µM), CDV (100 µM), FPV (100 µM), and a combination of 3TC, AZT and TFV (5 µM each) on trVLP infection of 293T cells (Fig. 4.3). Specifically, the 293T cells were treated with the different drugs at four different times relative to infection with trVLP, as indicated. We provide evidence that for each of the individual drugs and for the triple drug combination, at the doses indicated, trVLP infection of 293T cells is inhibited when treatment is initiated at +2, +6 or +24 hours post-infection. Interestingly, TOR, an estrogen receptor modulator discovered in a high throughput screen as a potent inhibitor of Ebola [25], significantly reduced viral luciferase activity at all time-points tested. For IFN-α, IFN-β, TOR and FPV treatments, maximal inhibition of trVLP infection was achieved when the cells were treated prior to challenge with trVLP. By contrast, pre-treatment with CDV at 100 µM, 24 hours prior to infection with trVLP, resulted in enhanced infection.
Fig. 4.2: IFNs and nucleoside analogues inhibit Ebola mini-genome replication \textit{in vitro.} (A) 293T cells were either left untreated (-), transfected with Tim-1 and the viral support protein plasmids NP, VP30, VP35, but not L (-L), or transfected with Tim-1 and all support plasmids that permit Ebola mini-genome entry and replication (+). At -24, 0, and +24 hours relative to infection, cells were treated with 5 μM of maraviroc (MVC), lamivudine (3TC), zidovudine (AZT), or tenofovir (TFV), or combinations of the three nucleoside analogues. Luciferase activity was measured four days after infection with trVLPs. (B) Luciferase activity of trVLP producer cells (0), first passage of 293T cells (1) treated with nucleoside analogues, and second passage of 293T cells (2) treated with nucleoside analogues. (C) Luciferase activity of 293T cells after treating with 5 μM combination of nucleoside analogues, cidofovir (CDV) or favipiravir (FPV). (D) Luciferase activity of 293T cells treated with IFN-α or IFN-β. The values are means of four biological replicates and are representative of two independent experiments. Error bars are the standard error of the mean. All drug treatment outcomes were statistically compared with the (+) control group in panels A, C and D.
Fig. 4.3: IFNs, toremifene, and nucleoside analogues inhibit trVLP replication. 293T cells were either left untreated (-), transfected with Tim-1 and the nucleocapsid plasmids NP, VP30, VP35 but not L (-L), or transfected with Tim-1 and all expression plasmids to permit Ebola minigenome entry and replication (+). (A-F) Cells were treated with the indicated drugs at the indicated doses, at each of the time points shown. Luciferase activity was measured 4 days post-trVLP infection. Values shown are the means of 4 biological replicates and are representative of 2 independent experiments. Error bars are the standard error of the mean. All drug treatment outcomes were statistically compared with the (+) control group.
In subsequent dose-response studies, we compared the inhibitory effects of IFN-α, IFN-β, TOR, CDV, FPV, 3TC, AZT or TFV when administered 24 hours post trVLP infection (Fig. 4.4). The data in Figure 4.4I summarize the IC$_{50}$ dose for each drug. The IFNs exhibited the lowest IC$_{50}$ values at 0.016 µM for IFN-β and 0.038 µM for IFN-α. The data show a log-fold difference in IC$_{50}$ values for IFN-α and IFN-β when compared in terms of U/ml, the norm for antiviral activity measurements (Figure 4.4 A, B). TOR had the next lowest IC$_{50}$ (0.36 µM) and completely inhibited infection at doses > 5 µM (Figure 4.4C). TFV had an IC$_{50}$ at 0.98 µM. CDV, 3TC and AZT all exhibited similar IC$_{50}$ values in the dose range 4.2-7.8 µM, while FPV had the highest IC$_{50}$ of the nucleoside analogues at 36.8 µM. At their IC$_{50}$ concentration, none of these drugs directly inhibited luciferase reporter activity (Fig. 4.5). We observed a relatively small antiviral dose range for CDV (1.5-25 µM) (Figure 4.4D), beyond which the drug appeared to enhance viral infection (Fig. 4.6). In cell viability assays we observe that at doses >10 µM CDV affects cell viability, confounding the interpretation of the effects of CDV on viral replication.
Fig. 4.4: IFNs, toremifene and nucleoside analogues administered 24 hrs post-exposure inhibit Ebola-mini-genome replication. 293T cells were either left untreated (-), transfected with Tim-1 and the nucleocapsid plasmids NP, VP30, VP35 but not L (-L), or transfected with Tim-1 and all expression plasmids to permit Ebola mini-genome entry and replication (+), as described in Materials and Methods. Twenty-four hours post-trVLP infection, cells were either left untreated, or treated with the indicated drugs (A-I) Dose-response plots for each of the indicated drugs. Luciferase activity (black circles) or cell viability (white squares) was measured 4 days post-infection (3 days after drug treatment). Values are the means of 4 biological replicates and are representative of 2 independent experiments. Error bars are the standard error of the mean.
Fig. 4.5: No direct effect of drugs on Renilla luciferase reporter assay. 293T cells transfected and then infected with trVLP were lysed 4 days post-infection. Cell lysate suspended in CCLR reagent was aliquoted into separate tubes and spiked with each drug at the IC₅₀ dose from Fig. 4.41 or DMSO solvent (+). Luciferase activity was then quantified. Values are the means of 3 independent experiments. Error bars are the standard error of the mean.
Fig. 4.6: CDV treatment increases trVLP replication. 293T cells were transfected with all expression plasmids that permit Ebola mini-genome entry and replication, then treated with the indicated doses of CDV 24 hrs post-trVLP infection. Luciferase activity was measured four days after cells were infected (3 days post-CDV treatment). Values are the means of 4 biological replicates and are representative of 2 independent experiments. Error bars are the standard error of the mean. CDV treatment outcomes were compared with the (+) control group.
In an orthogonal assay to confirm these findings, we next measured viral replication and transcription by qRT-PCR, following trVLP infection. trVLP-infected cells were either left untreated, or treated with the different drugs 24 hours post-infection, then viral replication and transcription evaluated 24 hours later (Fig. 4.7). All treatments, with the exception of TOR, significantly reduced the amount of genomic vRNA detected within cells (Figure 4.7A) and all treatments significantly reduced the synthesis of cRNA and mRNA isolated from infected cells (Figure 4.7B). Notably, IFN-β treatment of trVLP-infected cells resulted in the greatest reduction in viral replication and transcription.
Fig. 4.7: IFNs, toremifene and nucleoside analogues reduce trVLP replication and transcription. 293T cells were transfected with support plasmids and infected with trVLP. Twenty-four hours post-infection, cells were treated with the indicated drugs at their IC₅₀ doses, determined from Figure 2I. At 48 hrs post-infection, total RNA was extracted, reverse transcribed, then quantified by qPCR. Relative fold-change in negative sense vRNA transcripts (A) and positive sense cRNA and mRNA (B) was compared with infected, untreated cells (solvent, + control). Technical duplicates were examined by qPCR, and means are the average of three biological replicates. Error bars are the standard error around the mean.
Next we examined the effectiveness of two and three drug combinations on trVLP infection. We first examined 28 two-drug combinations, using each drug at its luciferase IC$_{50}$ value, and used the median-effect equation and combination index theorem [533] to determine drug synergy, additive or sub-additive effects (Figure 4.8A). Synergy is defined as greater than additive effect when drugs were combined (CI<1), additive as the effect expected when combining each drug (CI=1) and sub-additive as a smaller than expected additive effect (CI>1). Fractional inhibition (Fi) is defined as the percent reduction in luciferase activity. When administered 24 hours post-infection, many of the two-drug combinations showed strong synergism in inhibiting trVLP replication (Figure 4.8J), with IFN-β + 3TC demonstrating the greatest synergism (97.3% inhibition, CI = 0.028). 3TC was synergistic with all seven other drugs tested. Notably, when CDV was used in combination with FPV, AZT, TFV or IFN-α, it produced a sub-additive effect.

Next we tested all possible 56 three-drug combinations, using each drug at its IC$_{50}$ value, to assess whether adding a third drug enhanced efficacy compared with two-drug combinations (Figure 4.8B-I). This series of experiments served to validate our two-drug findings, as synergistic two-drug combinations such as IFN-β + 3TC and IFN-β + AZT, predicted strong synergy for the triple drug combination of IFN-β + 3TC + AZT. As anticipated from the two-drug polygonogram, CDV was sub-additive when combined in three-drug combinations (Figure 4.8E). This was most evident even when CDV was administered in conjunction with two-drug combinations that had shown strong synergy, such as IFN-β + 3TC or FPV + TFV, further indicating that CDV diminishes the antiviral effects of other drugs. IFN-β, 3TC, AZT and TFV all promoted strong synergism when included in triple drug combinations, with IFN-β + AZT specifically providing strong synergism when combined in three unique triple therapies.

From these two-drug and three-drug screens, we calculated the combination index (CI) and fractional inhibition (Fi) (Figure 4.8J-K). Many of the synergistic drug combinations (i.e. low CI) included one nucleoside analogue and an IFN, while those drug combinations that were sub-additive all included CDV. IFN-β was predominant in the most efficacious two- and three-drug combinations. In particular, IFN-β + 3TC and IFN-β + 3TC + AZT consistently exhibited the strongest synergism and highest Fi when administered 24 hours post-infection. Refer also to Tables 4.1 and 4.2.
Fig. 4.8: 2 and 3 drug combinations synergistically inhibit Ebola trVLP infection. 293T cells were either left untreated, transfected with Tim-1 and the support plasmids NP, VP30, VP35, but not L, or the mini-genome plus all the support plasmids. (A) Polygonogram of 28 two-drug combinations (at monotherapy IC$_{50}$ doses) administered at 24 hrs post-trVLP infection, then luciferase activity evaluated 3 days later. A thick red line represents strong synergy between two drugs (CI$<1$), a thin black line represents additive effects (CI=1), and a thick blue line represents strong sub-additive (less than additive) between two drugs (CI$>1$). (B-I) Polygonograms of 56 three-drug combinations. The backbone drug in each triple combination is listed above the heptagon, and the synergism/sub-additive effect of the additional two drugs is represented within each heptagon. (J-K) Combination index (CI) vs. fractional inhibition (Fi, percent luciferase inhibition) plots of the most synergistic and sub-additive double (J) and triple (K) drug combinations on trVLP luciferase activity. Dotted lines identify thresholds of synergy/additive effects. Values shown are the means of 2–4 biological replicates in 2 independent experiments. Error bars are the standard error of the mean.
Table 4.1: Fi and CI values for two-drug combination treatments. Experimental details are as described in the legend to Figure 4.8. The fractional inhibition (percent luciferase inhibition), combination index and strength of synergy (+), additive effects (+/-) or sub-additive (-) of each 2 drug combination therapy are shown.

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>Fractional Inhibition, Fi</th>
<th>Combination Index, CI</th>
<th>Synergism</th>
</tr>
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<tbody>
<tr>
<td>IFN-β + 3TC</td>
<td>0.973</td>
<td>0.028</td>
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<td>IFN-β + AZT</td>
<td>0.926</td>
<td>0.092</td>
<td>++++</td>
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<td>TOR + 3TC</td>
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<td>0.239</td>
<td>+++</td>
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<tr>
<td>FPV + TFV</td>
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<td>0.345</td>
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Table 4.2: Fi and CI values for three-drug combination treatments. Experimental details are as described in the legend to Figure 4.8. The fractional inhibition (percent luciferase inhibition), combination index and strength of synergy (+), additive effects (+/-) or sub-additive (-) of each 3 drug combination therapy are shown.

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>Fractional Inhibition, Fi</th>
<th>Combination Index, CI</th>
<th>Synergism</th>
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<td>0.881</td>
<td>0.486</td>
<td>+++</td>
</tr>
<tr>
<td>TOR + FPV + 3TC</td>
<td>0.877</td>
<td>0.517</td>
<td>+++</td>
</tr>
<tr>
<td>TOR + 3TC + TFV</td>
<td>0.858</td>
<td>0.520</td>
<td>+++</td>
</tr>
<tr>
<td>IFN-β + FPV + 3TC</td>
<td>0.809</td>
<td>0.534</td>
<td>+++</td>
</tr>
<tr>
<td>IFN-β + TOR + CDF</td>
<td>0.877</td>
<td>0.540</td>
<td>+++</td>
</tr>
<tr>
<td>IFN-α + 3TC + AZT</td>
<td>0.802</td>
<td>0.565</td>
<td>+++</td>
</tr>
<tr>
<td>IFN-α + TOR + 3TC</td>
<td>0.857</td>
<td>0.582</td>
<td>+++</td>
</tr>
<tr>
<td>CDF + 3TC + AZT</td>
<td>0.798</td>
<td>0.586</td>
<td>+++</td>
</tr>
<tr>
<td>IFN-α + CDF + TFV</td>
<td>0.789</td>
<td>0.599</td>
<td>+++</td>
</tr>
<tr>
<td>TOR + FPV + AZT</td>
<td>0.841</td>
<td>0.681</td>
<td>+++</td>
</tr>
<tr>
<td>TOR + FPV + TFV</td>
<td>0.830</td>
<td>0.684</td>
<td>+++</td>
</tr>
<tr>
<td>TOR + CDF + FPV</td>
<td>0.845</td>
<td>0.714</td>
<td>++</td>
</tr>
<tr>
<td>FPV + AZT + TFV</td>
<td>0.757</td>
<td>0.729</td>
<td>++</td>
</tr>
<tr>
<td>IFN-β + TOR + FPV</td>
<td>0.823</td>
<td>0.746</td>
<td>++</td>
</tr>
<tr>
<td>FPV + 3TC + AZT</td>
<td>0.752</td>
<td>0.809</td>
<td>++</td>
</tr>
<tr>
<td>TOR + CDF + AZT</td>
<td>0.808</td>
<td>0.835</td>
<td>++</td>
</tr>
<tr>
<td>IFN-β + 3TC + TFV</td>
<td>0.708</td>
<td>0.846</td>
<td>++</td>
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</table>
In a final series of experiments, in order to validate our findings from the trVLP infection studies, we examined the antiviral effectiveness of IFN-β, IFN-α, TOR, FVP, AZT, 3TC and TFV in 293T cells infected with ZEBOV (ZEBOV contained an eGFP reporter). CDV was excluded from these experiments. Initial dose-response studies were conducted at doses reflective of those used in the trVLP experiments in Figure 4.4. A higher dose of each drug was required to inhibit ZEBOV infection compared with trVLP infection (Fig. 4.9). Using the IC_{25} of each drug, we next evaluated 2 and 3 drug combinations for additive or synergistic effects against ZEBOV infection. All seven 2 drug combinations were synergistic (low CI) (Fig. 4.10A), similar to the most synergistic combinations against trVLP in Figure 4.8J. IFN-β + 3TC proved to be the most synergistic 2 drug combination, analogueous to trVLP infection. Of the most synergistic 3 drug combinations identified in the trVLP infection system, all seven exhibited synergy against ZEBOV infection, with IFN-β + 3TC + AZT and IFN-β + TOR + AZT exhibiting the strongest synergy (Figure 4.10B). The CIs determined from trVLP infection correlated well with those determined using ZEBOV infection; specifically, the correlation coefficients (R^2 values) confirm this (Figure 4.10C-D).

<table>
<thead>
<tr>
<th>Combination</th>
<th>CI 0.25</th>
<th>CI 0.50</th>
<th>Result</th>
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<tr>
<td>TOR + 3TC + AZT</td>
<td>0.783</td>
<td>0.893</td>
<td>+</td>
</tr>
<tr>
<td>CDF + FPV + AZT</td>
<td>0.742</td>
<td>0.909</td>
<td>(+/-)</td>
</tr>
<tr>
<td>CDF + FPV + 3TC</td>
<td>0.722</td>
<td>0.966</td>
<td>(+/-)</td>
</tr>
<tr>
<td>IFN-β + CDF + TFV</td>
<td>0.679</td>
<td>0.982</td>
<td>(+/-)</td>
</tr>
<tr>
<td>IFN-α + CDF + AZT</td>
<td>0.712</td>
<td>1.019</td>
<td>(+/-)</td>
</tr>
<tr>
<td>IFN-α + IFN-β + CDF</td>
<td>0.697</td>
<td>1.033</td>
<td>(+/-)</td>
</tr>
<tr>
<td>IFN-β + CDF + AZT</td>
<td>0.685</td>
<td>1.088</td>
<td>(+/-)</td>
</tr>
<tr>
<td>IFN-α + FPV + TFV</td>
<td>0.666</td>
<td>1.202</td>
<td>--</td>
</tr>
<tr>
<td>IFN-α + TOR + FPV</td>
<td>0.712</td>
<td>1.350</td>
<td>--</td>
</tr>
<tr>
<td>IFN-α + CDF + 3TC</td>
<td>0.620</td>
<td>1.489</td>
<td>---</td>
</tr>
<tr>
<td>TOR + CDF + TFV</td>
<td>0.649</td>
<td>1.524</td>
<td>---</td>
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<tr>
<td>IFN-β + CDF + FPV</td>
<td>0.613</td>
<td>1.527</td>
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<td>0.641</td>
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<td>0.597</td>
<td>1.563</td>
<td>---</td>
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<tr>
<td>IFN-β + CDF + 3TC</td>
<td>0.589</td>
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<td>IFN-α + TOR + CDF</td>
<td>0.643</td>
<td>1.723</td>
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<tr>
<td>CDF + FPV + TFV</td>
<td>0.468</td>
<td>2.616</td>
<td>---</td>
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<td>IFN-α + CDF + FPV</td>
<td>0.445</td>
<td>3.153</td>
<td>---</td>
</tr>
<tr>
<td>CDF + 3TC + TFV</td>
<td>0.380</td>
<td>3.804</td>
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Fig. 4.9: IFNs, toremifene and nucleoside analogues administered 24 hrs post-exposure inhibit ZEBOV-GFP. (A-H) 293T cells were infected with ZEBOV-GFP (MOI = 0.1). Twenty-four hours post-infection cells were either left untreated, or treated with the indicated drugs at the indicated doses. Intracellular GFP was measured 48 hours later and the percent inhibition quantified relative to infected, untreated cells (DMSO solvent control). Values are the means of 4 biological replicates. Error bars are the standard error of the mean.
**Fig. 4.10:** Synergistic 2 and 3 drug combinations against trVLP-LUC infection inhibit fully infectious ZEBOV-GFP. (A-B) 293T cells were infected with ZEBOV-eGFP at an MOI of 0.1, then treated with 2 and 3 drug combinations as indicated, 24 hours post-infection, at their monotherapy IC$_{25}$ doses. GFP fluorescence was measured 3 days post-infection. Values are the means of 4 biological replicates, and error bars represent the standard error of the mean. Data are representative of 2 independent experiments. The combination index (CI) was plotted against the fractional inhibition (Fi, percent GFP inhibition) for each drug combination. (C-D) Plot of CIs for ZEBOV infections compared with CIs for trVLP infections.
4.5 Discussion

In September 2014, the WHO hosted a conference to facilitate development of a global action plan to deal with the Ebola outbreak in West Africa. Delegates from affected West African countries, ethicists, scientists, health care providers, logisticians and representatives from different funding agencies were in attendance. A committee had been struck to evaluate the different vaccine candidates and therapeutic interventions being developed, which subsequently received an overwhelming number of submissions for consideration, and was hampered by an inability to compare antiviral effectiveness, since in vitro and pre-clinical in vivo model systems vary, treatment regimens vary from prophylaxis to post-exposure administration, and direct readouts of antiviral efficacy differ. Moreover, given the virulence and high mortality associated with EVD, all of these studies have been conducted under BSL 4 conditions, limiting the number of laboratories that can engage in these antiviral studies. Cognizant of these limitations, we employed the trVLP model system to compare the antiviral effectiveness of eight antiviral candidates from three drug classes. We evaluated their antiviral activities in the context of inhibition of Ebola replication, using this mini-genome model that allows for rapid comparisons among compounds under BSL 2 conditions. The tetracistronic minigenome represents the most sophisticated in vitro replication model of Ebola virus to date. trVLPs proceed through every replication step as wild type Ebola virus, and have been tested in multiple cell lines. Using TOR, there has been some validation of the trVLP assay. Specifically, TOR has been evaluated in limiting Ebola virus infection of VeroE6 and HepG2 cells, and exhibited IC\textsubscript{50} values of 0.2 µM and 0.03 µM, respectively [586], in line with the IC\textsubscript{50} dose for TOR (0.36 µM) observed with trVLP infection. Likewise, the IC\textsubscript{50} identified in the trVLP system for FPV (36.8 µM), is consistent with that of 67 µM recorded using Ebola virus infection [23], suggesting that this Ebola mini-genome system has relevance for screening potential antiviral compounds. Indeed, our validation studies using ZEBOV (ZEBOV-eGFP) suggest that the trVLP infection model has utility as an in vitro screening assay when comparing different drugs as monotherapies or in 2 and 3 drug combinations.
As mentioned, the Ebola virus encodes in its genome factors that limit a type I IFN response to infection [577-579]. Yet, both rodent and non-human primate studies suggest that IFN-α and IFN-β treatment can confer partial protection from infection, reducing viremia and prolonging survival [31, 580, 581], suggesting that it may be possible to override the inhibitory effects of the virus by treatment with IFN. At the outset, we conducted a series of experiments to compare the antiviral activities of IFN-α and IFN-β in the trVLP infection system, and our findings suggest that whether treatment is administered prior to or post-infection, both IFN-α and IFN-β exhibit antiviral activity. These findings only have relevance for the direct antiviral activities of these IFNs, since the effects of IFN-α or IFN-β on immune modulation for viral clearance cannot be determined using this system. Nevertheless, these data contributed to the decision to conduct a clinical trial of IFN-β treatment for EVD in Guinea.

We provide evidence that the nucleoside/nucleotide analogues 3TC, AZT, TFV, FPV and CDV inhibit Ebola trVLP replication in vitro. The results with 3TC are in contrast to published data that show no evidence for 3TC inhibiting Ebola virus infection in vitro [519]. These studies examined the antiviral effectiveness of 3TC when administered one hour prior to infection, in contrast to our studies that have focused on post-exposure protection. In cells, the kinetics of 3TC phosphorylation are such that a minimum of four hours are required for optimal activity, perhaps distinguishing why our 24 hour pre-treatment, specifically a combination treatment, offered protection. Post-exposure treatment with 3TC and the other nucleoside/nucleotide analogues we examined, would more likely reveal activity against viral RNA synthesis than pre-treatment. When comparing the IC\textsubscript{50} values of each of the nucleoside analogues that we tested, TFV exhibited the lowest IC\textsubscript{50} at ~1 µM. Whether this reflects the fact that this adenosine monophosphate analogue only requires two phosphorylation events to become an active drug versus three for the other nucleoside analogues, remains undetermined. Extensive published data reveal both the safety profiles [523, 587, 588] and the biodistribution of 3TC, AZT and TFV in the circulation and liver [416, 538], the same compartments where Ebola infects monocytes, macrophages, dendritic cells, endothelial cells and hepatocytes. Moreover, drug interactions with other nucleoside analogues have been well studied: e.g. tenofovir disoproxil fumarate, when
used alone or in combination with emtricitabine effectively prevents HIV-1 infection in antiretroviral pre-exposure prophylaxis (PrEP) [588].

Our studies also revealed that the active metabolite of brincidofovir, CDV, has a narrow therapeutic window of efficacy (6.25-25 µM) when assessed in the trVLP assay, enhancing viral replication at higher doses when added either prior to or post-infection. In cell viability assays, CDV exhibits cytotoxicity at doses >10 µM. These findings suggest that caution is required if CDV is to be considered further for the treatment of EVD, specifically that phase I/II trials define the safety profile of this drug for EVD.

Another advantage of this in vitro system is that it allowed us to evaluate various 2 and 3 drug combinations and demonstrates that combination treatments limit viral replication up to 97.3%. A benefit of combination treatment is the potential to limit/avoid the emergence of drug resistance. Interestingly, IFN-β was predominant among all the 8 antivirals considered in terms of contributing very strong synergism in combination treatments: e.g. IFN-β + 3TC; IFN-β + 3TC + AZT. Using this system, we observe that FPV, when administered 24 hours post-infection, has an IC_{50} of ~ 37 µM. To date, the phase II/III JIKI trial examining the efficacy of FPV against EVD has reported only modestly encouraging results. In our 2 drug combination treatment studies we show that, with the exception of CDV, whenever FPV is included, synergy occurs, effectively reducing the CI. It may transpire that for treating EVD, FPV is most effective in a drug combination regimen.

Viewed altogether, we present an in vitro Ebola trVLP screening system, that requires only level 2 biocontainment, which allowed us to compare the antiviral activities of 8 compounds, either alone or in combination. We provide evidence that IFNs are effective inhibitors of Ebola replication, with IFN-β exhibiting greater efficacy over IFN-α, or when used in combination with nucleoside analogues. We infer from our data that whether IFN-β treatment is administered 24 hours prior to, or up to 24 hours post-infection, reduced Ebola replication is achieved. As additional antiviral therapeutic candidates become available, we now have the capability to measure and compare their direct antiviral activities with the existing panel. This allows for rapid
*in vitro* evaluation and the opportunity to prioritize antiviral candidates for further pre-clinical and clinical trial studies.
Chapter 5: Key Findings, Future Perspectives, Conclusions and Broader Significance
5.1 Key Findings, Limitations and Future Perspectives

HIV-1

As new indications for small-molecule kinase inhibitors expand beyond the treatment of various cancers [169, 170], the essential role of non-receptor tyrosine kinases facilitating viral replication are increasingly being recognized, along with their associated disease pathologies that can be targeted with small-molecule inhibitors [146, 178, 201, 554]. At the outset, this work investigated the role of the SFK member c-SRC during early HIV-1 infection, to determine whether it has particular signaling functions during early HIV-1 replication in diverse CD4+ T-cell lines and primary human CD4+ T-cells. A variety of techniques were used, including SFK tool-drug compounds PP1, PP2 and SU6656, ADV vector overexpressing DN c-SRC, and targeted siRNA knockdown (chapter 2). It was also determined whether the focal adhesion kinase binding partner PTK2B exhibited similar affects as c-SRC during early HIV-1 infection, and a variety of HIV-1 viruses were considered, including: X4- or R5-tropic viruses, nef-deficient or fully infectious viruses, and laboratory strains versus primary clinical isolates. The essential role of c-SRC during early time-points of HIV-1 infection led to further exploration of four potent c-SRC kinase inhibitors, providing evidence to support further testing of these preclinical or FDA-approved compounds for further studies of HIV-1 inhibition (chapter 3).

Lastly, the work presented in this thesis expanded on the use of FDA-approved drugs, used to treat HIV-1, for repurposing for use in EVD. Monotherapy and combination antiviral therapy of 8 antiviral candidates for inhibition of a ZEBOV mini-genome model of replication or in vitro ZEBOV infection were used (chapter 4). Nucleoside analogues that were used for compassionate EVD treatment during the 2014-16 ZEBOV outbreak in West Africa were a particular focus [519]. The trVLP model of ZEBOV replication permitted rapid assessment of drugs and combinations of therapies to prioritize testing of compounds, leading to confirmatory BLS4 testing of the most synergistic drug combinations, and providing rationale for future drug development in small animal models of EVD.

Prior to this work, the roles of LCK and FYN signaling had been the most studied in HIV-1 infection of CD4+ T-cells [227, 238], but an understanding of a role for c-SRC signaling had not
been clearly defined. Early LCK activation downstream of CD4 can be decoupled from the TCR-CD3 complex by Nef and impair formation of the immunological synapse [589]. LCK also becomes recycled with CD4 at endosomes, contributes to Tat-mediated HIV-1 transcription, facilitates Gag-assembly during viral egress, and promotes virological synapse transmission of HIV-1 [222, 226-228, 589]. Activated FYN signaling promotes HIV-1 transcription through NF-κB, enhances assembly and release of virions, and yet becomes inhibited by viral Vif to reduce phosphorylated APOBEC3G packaged into budding virions [227, 237, 238]. Similar to both LCK and FYN, this work demonstrates that c-SRC becomes activated and phosphorylated at Tyr419 within the first hour of HIV-1 IIIB (X4) or HXB2 (X4) infection of Jurkat C T-cells (chapter 2), or HXB2 (X4) or JR-FL (R5) infection of activated primary CD4+ T-cells (chapter 3). This was strongest for virus expressing Nef, but not completely absent in pseudo-enveloped viruses lacking Nef (HXB2 or JR-FL), which may suggest c-SRC activation is partially independent of Nef in the first hour of CD4+ T-cell infection. Similar results were found for the binding partner PTK2B, where its phosphorylation at Tyr402 increased after 1 hr of HIV-1 IIIB infection in Jurkat C T-cells, or HXB2 or JR-FL infection in activated CD4+ T-cells. However, PTK2B was not phosphorylated at Tyr402 after 1 hr of HXB2 infection in Jurkat C cells. This was the first of many indications that signaling within T-cell lines may not be the best models to recapitulate physiological signaling cascades of c-SRC or PTK2B in primary CD4+ T-cells.

In chapter 2, it was demonstrated that pan-inhibition of SFKs with 10-20 μM of the tool compound SU6656 significantly enhanced HIV-1 luciferase activity of nef-deficient VSV-G/HIV-1 in Jurkat C or Jurkat E6-1 infection. This observation correlated with decreased in vitro c-SRC kinase activity and no change in cell viability. The SU6656 experiments were corroborated with follow-up tests of DN c-SRC ADV vector overexpression, which increased VSV-G/HIV-1 infection in Hut 78 and Kit 225 cells. However, these ADV results should be interpreted cautiously, as ADV treatment alone caused high cell death in Jurkat E6-1 and Hut 78 cells (70-80% of cells were 7-AAD+), and EYFP transfection efficiency was not equivalent between cell lines. These findings suggest further optimization of ADV vector MOI is needed in the three T-cell lines. Future experiments should also evaluate whether a dominant positive (DP) mutant of c-SRC decreases VSV-G/HIV-1 infection in T-cell lines, as would be predicted. This
work from chapter 2 also revealed that siRNA reduction of c-SRC or PTK2B expression in Jurkat E6-1 cells increased HXB2 reverse transcription. The pools of 4 siRNAs were kinase-specific, had no affect on cell viability when administered by lipofection, nor change in surface expression of CD4 or CXCR4 (chapter 2). The increased HXB2 luciferase activity from c-SRC or PTK2B siRNA knockdown also correlated with increased early and late reverse transcript cDNA, as well as 3-4-fold higher integrated virus after 24 hrs of infection.

However, these drug and siRNA findings are in contrast with the results of primary CD4+ T-cells studied in chapter 3, and the published research of others describing a protective effect of c-SRC inhibition or PTK2B siRNA knockdown 6 days post-infection [264, 345]. Different SFK inhibitors were employed in chapters 2 and 3 that inhibit different off-target kinases. SRC Inhibitor-1 is reported to be the most selective for SFKs at 1 μM in an in vitro study of 73 human kinases (4 off-targets), followed by PP1 (5 off-targets), PP2 (5 off-targets) and SU6656 (9 off-targets) [557]. In comparison, dasatinib, saracatinib and KX2 391 are less selective inhibitors of SFKs, targeting several other kinases such as ABL1, ARG and serine/threonine kinases [590, 591]. Yet employing the same pool of siRNA to knockdown c-SRC in chapters 2 and 3 caused opposite changes to luciferase activity in HIV-1 infected Jurkat cells compared with primary CD4+ T-cells, suggesting the observed differences are primarily caused by the cell type investigated.

Taken together, these findings cast doubt on the generalizability of c-SRC and PTK2B signaling in transformed T-cell lines, with their corresponding signaling events in primary human CD4+ T-cells during HIV-1 infection. Indeed, a comprehensive study on the proximal TCR signaling in Jurkat E6-1 and Hut 78 cells, relative to activated primary CD4+ T-cells, showed that upon TCR stimulation the cell lines exhibited greater Ca^{2+} flux, uncharacteristic expression of costimulatory receptors, atypical cytokine release, and hyperphosphorylation of PTK2B and c-SRC, among other downstream signal transduction abnormalities (see Figure 5.1, originally published in [562]). Thus, while mutant T-cell sublines can be useful in other contexts [592], the work in this thesis shifted instead to focus on the role of early c-SRC and PTK2B signaling in HIV-1 infection of primary CD4+ T-cells, isolated from healthy human donors (chapter 3).
Fig. 5.1: Aberrant TCR signaling in Jurkat T-cells. PTK2B (Pyk2) and other proximal signaling kinases are hyperphosphorylated in Jurkat E6-1 compared with primary activated CD4⁺ T-cells. Overexpression or hyperphosphorylation are green, while underexpression or hypophosphorylation are in red [562].
In activated primary CD4+ T-cells, this work demonstrates that c-SRC co-immunoprecipitates with a truncated PTK2B fragment phosphorylated at Tyr402, 1 hr following infection of nef-deficient HXB2 or JR-FL virus (chapter 3). To explain this finding, HIV-1 chemokine receptor binding may induce cleavage of PTK2B, potentially through CXCR4, CCR5, or P2Y2 activation and signaling [147, 345]. This highlights a potential PTK2B similarity with closely related FAK during HIV-1 infection. Downstream of gp120/CD4 signaling, phosphorylated FAK becomes cleaved by caspase-3 and caspase-6 into a 90 kDa fragment containing the FAT domain, and a 35 kDa fragment containing the kinase domain of FAK [322, 346]. Separating the FAT domain from focal adhesions in this manner has been shown to promote p53-dependent apoptosis [347, 348]. Thus, it should be explored in future experiments whether during HIV-1 infection similar signaling from the larger PTK2B fragment containing the FAT domain occurs, as the smaller, phosphorylated kinase fragment of PTK2B binds to c-SRC.

To investigate the effects of activated c-SRC and PTK2B on the early stages of HIV-1 replication, careful optimization of siRNA electroporation was performed, as CD4+ T-cells are challenging non-adherent cells to transfect (chapter 3). Moreover, activated primary CD4+ T-cells degrade argonaute, an enzyme essential to the RNA-Induced Silencing Complex (RISC) [593]. Without argonaute, many mRNAs become de-repressed, which promotes rapid protein translation as part of normal T-cell activation and T-cell differentiation [593]. This natural phenomenon of argonaute degradation upon activation therefore precluded testing of c-SRC or PTK2B siRNA knockdown after T-cell activation. Primary CD4+ T-cells administered siRNA, and then activated, showed no changes to CD4, CXCR4 or CCR5 expression, or reduction in cell viability (chapter 3).

The findings in chapter 3 reveal that viral reverse transcription of either HXB2 or JR-FL was unaffected by c-SRC or PTK2B siRNA knockdown. However, reducing c-SRC expression in CD4+ T-cells prior to infection with nef-deficient HXB2 or JR-FL viruses, increased the detection of early reverse transcripts, late reverse transcripts, and 2-LTR circles, while reducing viral integration and luciferase reporter activity. Similar results were found after PTK2B siRNA knockdown, although only during JR-FL (R5) infection. This suggests PTK2B activation is a byproduct of HXB2 (X4) and IIIB (X4) entry, perhaps through CXCR4 stimulation [147], and is
not essential in the early stages of HXB2 or IIIB replication in an activated CD4\(^+\) T-cell. While both c-SRC and PTK2B are activated upon HIV-1 binding and fusion, they could be transmitting signals that prepare the cell for later stages of the intracellular virus replication cycle [147]. To generalize these c-SRC and PTK2B findings to multiple strains of HIV-1, testing of more X4 and R5 HIV-1 clinical isolates are needed. Nonetheless, infection with HIV-1 strains expressing Nef (IIIB (X4), Ba-L (R5) or KNH1207 (R5)) could not rescue infection after c-SRC siRNA knockdown, as quantified by qPCR integration and p24 ELISA measurements, suggesting the function of c-SRC may be independent of Nef during early infection. This outcome was not entirely surprising, given the low affinity of \textit{in vitro} c-SRC SH3 binding to Nef PxxP, relative to the stronger binding of HCK or LYN to Nef that is mediated by a key Ile residue in their SH3 domain [164]. Collectively, these results from chapter 3 suggest changing roles for c-SRC and PTK2B signaling at various time-points and stages of the early HIV-1 replication cycle, similar to SFKs LCK and HCK during HIV-1 infection [223, 227, 238, 249]. In future experiments, it will be of interest to test whether c-SRC or PTK2B siRNA knockdown increase HIV-1 reverse transcripts and reduce viral integration in a similar fashion in T-cell sub populations. These experiments could be performed in T-cells that maintain the viral reservoir in lymph nodes (central memory peripheral T-follicular cells), prevent microbial translocation at the gut and control inflammation at mucosal barriers (Th17 cells), or play a role in suppressing immune hyperactivation (Tregs) [80, 594, 595].

The increase in observed early and late reverse transcripts of JR-FL virus after c-SRC or PTK2B siRNA knockdown, together with no effect on RT activity, strongly suggest these kinases act after reverse transcription and prior to viral integration (chapter 3). Other kinases have shown similar effects on HIV-1, such as JNK inhibition increasing reverse transcripts and impairing viral integration [159]. Unlike LCK and FYN, c-SRC interacts with membrane proteins of multiple compartments in addition to microdomains at the plasma membrane [189], suggesting c-SRC could have roles in the cytosolic or nuclear stages of HIV-1 replication. Moreover, the SH3 domain of c-SRC regulates its localization from actin-associated focal adhesions to the perinuclear region in a microtubule-dependent manner [189]. The function of host and viral proteins that constitute the pre-integration complex of HIV-1 are still under investigation [155].
It also remains to be determined whether c-SRC or PTK2B directly phosphorylate host and viral proteins in the PIC, or bind PIC proteins through SH2 or SH3 interactions to stabilize the complex. In support of these hypotheses, c-SRC has been predicted by NetPhos 3.1 to phosphorylate HIV-1 RT at eight different sites in its protein sequence (Tyr56, Tyr115, Tyr188, Tyr342, Tyr354, Tyr405, Tyr441 and Tyr457) with 31-52% confidence, and three sites in the IN sequence (Tyr15, Tyr83 and Tyr99) with 37-48% confidence [596]. Comparing with dengue virus infection, it has been proposed the related family member FYN phosphorylates host factors in that RNA replication complex, or may act as an adaptor protein that scaffolds interactions between components of the dengue virus replication complex [178]. In addition, c-SRC has been shown to directly interact with the HCV RdRP complex during HCV infection, facilitating intracellular replication [202]. In the context of HCV replication, the SH3 domain of c-SRC is exploited to bind the viral polymerase NS5B, linking c-SRC to the non-structural phosphoprotein NS5A through its SH2 domain [202]. These c-SRC interactions were also found to be essential for NS5B and NS5A binding each other and HCV replication, and were not mediated by related SFK members FYN or c-YES [202]. Thus, c-SRC could have a novel and essential interaction within the PIC of HIV-1. This line of inquiry is part of future experiments that involve isolating the HIV-1 PIC, to determine a potential scaffolding role for c-SRC.

The buildup of 2-LTR circles and reduced proviral integration after c-SRC or PTK2B knockdown further implicate these kinases in the nuclear translocation of the HIV-1 PIC (chapter 3). Indeed, this work showed that in the presence of the integrase inhibitor raltegravir, the synthesis of HXB2 or Ba-L cDNA was similar to the nuclear effects of c-SRC or PTK2B siRNA knockdown. Others have reported similar increases in 2-LTR circles after raltegravir intensification in HIV-1 patients taking cART [565]. There is precedent for non-receptor kinases affecting the nuclear biology of HIV-1, such as MAPK phosphorylation of the inner nuclear lamina protein emerin (EMD) [154]. If EMD phosphorylation is blocked, or EMD expression silenced by siRNA, proviral integration decreases and 1- and 2-LTR circles accumulate [154]. The phosphorylation of nuclear proteins may very well be a common lentivirus mechanism for accessing the nuclear compartment [154], and may include c-SRC kinase activity during HIV-1 infection. c-SRC has been found to localize to the nucleus and nucleolus, and may regulate
proteins involved in cell cycle progression and entry into mitosis [189]. Tyr419 phosphorylation on nuclear c-SRC has been shown to induce c-SRC interactions with Sam68 in other cell types [597]. Thus nuclear c-SRC may already have a role in HIV-1 replication through Sam68, as Sam68 regulates HIV-1 RNA processing, the transactivation of Rev, viral mRNA nuclear transport, and mRNA translation in the cytosol [317]. Research into c-SRC protein interactions with perinuclear membrane and nuclear protein substrates is ongoing [189]. One hypothesis is that nuclear c-SRC could be regulating DNA ligase 4 (DNAL4), an essential DNA repair protein in the NHEJ pathway that specifically catalyzes the ligation of unintegrated HIV-1 cDNA into 2-LTR circles [155]. It has been put forth that the free ends of unintegrated HIV-1 cDNA are sensed as DNA double-strand breaks, which activate nuclear kinases that regulate DNA repair mechanisms to circularize viral cDNA, thus pre-empting apoptosis signaling by the T-cell [155]. Our lab is now investigating the potential role of c-SRC signaling in this NHEJ repair pathway that circularizes HIV-1 cDNA into 2-LTR circles during HIV-1 replication [598].

The findings in chapter 3 also demonstrate the novelty of four specific c-SRC inhibitors that strongly reduce JR-FL or HXB2 infection in activated CD4\(^+\) T-cells. Dasatinib, saracatinib, KX2-391 and SRC inhibitor-1 all inhibited the luciferase activity of these recombinant viruses, and caused little to no increase in apoptosis or necrosis. Dasatinib was the most potent of the four inhibitors examined, reducing HXB2 or JR-FL infection in the 10-100 nM range. This drug has recently been shown to inhibit the phosphorylation of SAMHD1, a c-SRC substrate [537]. Without phosphorylation of negative regulatory tyrosine residue Tyr592, SAMHD1 becomes activated and reduces the cytosolic pool of free dNTPs, impairing HIV-1 reverse transcription in dasatinib-pretreated primary CD4\(^+\) T-cells [537]. Dasatinib is a safe and well tolerated kinase inhibitor, approved for second-line treatment of CML [553]. Moreover, the related SRC/ABL inhibitor imatinib has been used to safely treat KS or CML cancers in HIV-1 patients on fully suppressive cART, improving long-term survival [176, 599, 600]. These clinical examples of kinase inhibitor provokes the question: Can dasatinib be used safely in conjunction with cART to further suppress HIV-1 infection? At doses within well-tolerated levels in humans, dasatinib has also been shown to inhibit the \textit{ex vivo} reactivation of HIV-1 from the CD4\(^+\) T-cells of treatment naïve, HIV-1 donors [554]. To investigate the mechanism of action of dasatinib, which targets
Abelson murine Leukemia viral oncogene homolog 1 (ABL1) and Abelson-Related Gene (ARG) kinases as well as c-SRC, the role of these two ABL family kinases during HIV-1 infection is another active area of future research. Furthermore, current investigations in our lab are expanding on the findings presented in chapter 3, to test whether dasatinib treatment can reduce \textit{in vivo} Ba-L infection in a humanized mouse model of HIV-1 infection. This model will be an excellent means of studying HIV-1 resistance mutations that may develop from weeks of dasatinib monotherapy, which could be pre-empted with combination therapy if they occur. We will also explore whether dasatinib can be included in cART as an alternative integration inhibitor in this mouse model of HIV-1 infection. Furthermore, CRISPR/Cas9 may be used to knockout c-SRC or PTK2B in human CD34$^+$ Hematopoietic Progenitor Cells (HPCs) prior to their transplantation into mice, to ascribe more definitive \textit{in vivo} roles of c-SRC and PTK2B signaling during HIV-1 infection.

**ZEBOV**

In September of 2014, during the height of the ZEBOV outbreak in West Africa and when the first documented case of ZEBOV transmission occurred in the United States [601], the need to rapidly assess drugs for preclinical ZEBOV antiviral activity was of paramount concern to the National Microbiology Laboratory of Canada, US CDC, USAMRIID, and the WHO Working Group on Ebola Therapeutics. By establishing a new trVLP model of ZEBOV replication in the lab [534], the four c-SRC inhibitors from chapter 3 were initially tested for activity against ZEBOV trVLP replication. While this SFK research was a novel avenue of its own, and preliminary results were promising, the urgent need to test and compare experimental therapies, either fast-tracked for upcoming phase II/III clinical trials in West Africa (BCV, FPV and IFN-β), or already being used for compassionate EVD treatment (AZT and 3TC), took precedent for \textit{in vitro} testing [29, 31, 431, 519]. Thus, 8 compounds from 3 drug classes were evaluated alone or in combination, to provide additional rationale for further preclinical development or their potential use in emergency ZEBOV clinical trials (chapter 4).
HEK 293T cells are an ideal cell line for the ZEBOV trVLP model because their high transfection rate produces many virions [534], and the cells can quickly convert nucleoside analogs to their active triphosphate form analagous to primary hepatocytes [418]. Indeed, VeroE6 show slower phosphorylation of nucleoside analogs and may under represent drug potency [418]. As 293T cells are not the natural target cell infected by ZEBOV, it was important to first validate the in vitro model with therapeutic treatments published to inhibit fully infectious ZEBOV in other cell lines [23, 586]. The ZEBOV GP1,2 entry inhibitor TOR showed very similar IC_{50} results in the trVLP model (0.36 μM) as with publications of TOR inhibiting ZEBOV in VeroE6 (0.2 μM) or HepG2 cells (0.03 μM) [586]. Similarly, FPV has been reported to inhibit ZEBOV in VeroE6 cells with an IC_{50} of 67 μM [23], comparable to the IC_{50} of 36.8 μM found for ZEBOV trVLP in chapter 4. Together, this data suggested the trVLP model of ZEBOV infection in 293T cells could be used to screen potential antiviral compounds.

As predicted from in silico simulations of RdRP L nucleotide analogue docking [456], 3TC, AZT and TFV each showed dose-dependent inhibition of ZEBOV trVLP luciferase activity, which was independent of whether the drug was administered a day before, during or one day after infection (chapter 4). TFV, which was predicted out of 20 nucleoside and nucleotide analogs to have the strongest interaction with the putative RdRP L nucleotide binding pocket [456], showed the lowest IC_{50} of the 5 nucleoside analogs tested (0.98 μM). This was followed by AZT (4.2 μM), 3TC (6.2 μM) and CDV (7.7 μM) giving similar doses for 50% inhibition. Interestingly FPV had the highest IC_{50} (36.8 μM). FPV is a drug that is required in high doses to protect small animals from lethal ZEBOV exposure [29]. It is also required in high doses in non-human primates to achieve target plasma levels [441]. Furthermore, FPV showed sub-optimal dosing in the blood samples of EVD patients in the recently completed phase II JIKI trial in Guinea [444]. From these multiple lines of investigation, it would appear FPV is a mild inhibitor of ZEBOV RdRP that could benefit from synergistic drug combinations to reduce the high doses of drug required in monotherapy.

The work presented in chapter 4 is the first account of 3TC, AZT or TFV having antiviral activity against ZEBOV trVLP replication or against ZEBOV-GFP in vitro infection in BSL4. As a potential mechanism of action, these three nucleoside/nucleotide analogues were then
shown to inhibit viral synthesis of negative-sense genomic vRNA and positive-sense cRNA and mRNA, as measured by qRT-PCR. TFV showed the strongest antiviral activity of the 5 nucleoside analogues tested in this qRT-PCR assay. At first, these findings appear to be in contrast with recent published work suggesting 3TC and AZT exhibit weak *in vitro* activity against ZEBOV replication [518]. However, the publication showed similarly mild dose/response curves of 3TC and AZT against ZEBOV-GFP, as reported in chapter 4. For instance, 25 μM of 3TC caused 18% inhibition of ZEBOV-Makona in their report [518], while the same 25 μM dose of 3TC caused 22% inhibition of ZEBOV-GFP in chapter 4. It was anticipated that the required drug doses would be higher when moving from the ZEBOV trVLP model to fully intact ZEBOV-GFP infection at an MOI of 0.1. Nevertheless, the primary value in testing 3TC and AZT came not from monotherapy alone, but their potential to synergize in the trVLP model of ZEBOV infection. In the top 7 two-drug and top 7 three-drug combinations demonstrating the strongest synergy against ZEBOV-GFP, 3TC was included in 5 of these combinations, and AZT was in 5 combinations as well (chapter 4). Likewise, the recent ZEBOV study testing 3TC and AZT also reported drug synergy when combining 12.5 μM of 3TC with 25 μM of AZT, markedly reducing ZEBOV-Makona infection by 70% [518]. To further clarify the mechanisms of 3TC and AZT ZEBOV inhibition, future work demonstrating RNA chain termination of RdRP L in the presence of either nucleoside analogue, and crystal structures of the RdRP L enzyme bound with either compound, would be desirable. In addition, the antiviral activity of 3TC and AZT in ZEBOV infection should be confirmed in primary monocyte-derived macrophages, to strengthen the preclinical evidence for evaluating these two drugs further in small animal models of ZEBOV infection.

Treating 293T cells with the metabolite of BCV (CDV) at or above 100 μM prior to or post-ZEBOV trVLP infection, unexpectedly and consistently increased viral luciferase activity 4 days post-infection (chapter 4). Although CDV inhibited trVLP luciferase activity in a small range of concentrations (1.25-25 μM), it reduced the antiviral activity of many of the other 7 drugs tested in two- or three-drug combinations. Others have reported BCV to enhance ZEBOV-GFP infection in Huh7 cells, at doses less than 0.2 μM [28]. In two recent phase III trials, BCV has failed to show efficacy in treating ADV infections or preventing CMV infection during
hematopoietic cell transplantation in patients. Additionally, a third phase III trial of BCV to prevent CMV infection after kidney transplant was recently terminated. BCV was originally created to be a less toxic alternative to CDV, and shows no associated nephrotoxicity in patients [602]. However, an oral BCV dose of 200 mg twice a week in a study of 30 participants was shown to elevate liver enzymes detected in blood samples, such as Alanine Transaminase (ALT) (40% of participants were > 3x upper limit of normal range) [413]. Elevated bloodstream levels of liver enzymes can indicate potential inflammation or injury to the liver [603]. Two hundred mg of BCV twice a week also caused diarrhea for 70% of the participants, leading to 60% of those taking the drug to withdraw from the study from adverse effects [413]. It is possible that in the BCV phase II ZEBOV trial, which only recruited 4 EVD patients of which none survived [24], BCV may have unintentionally exacerbated liver dysfunction and diarrhea symptoms. Evidence supporting this are: 1) Patient #2 in the BCV ZEBOV trial had hepatic injury (high ALT levels) and persistent diarrhea, leading to suspension of BCV treatment by day 7 [24]. 2) It was determined in the JIKI trial, which measured baseline viremia in 58 EVD patients and serum ALT over 25 days, that patients nearing death had elevated ALT levels compared with those who survived [18]. Collectively, these BCV findings and CDV results presented in chapter 4 highlight the necessity of establishing clear preclinical in vitro antiviral activity and in vivo animal efficacy in preventing or treating lethal ZEBOV challenge, prior to initiating a phase II trial during an epidemic setting [24].

The last key finding from chapter 4 was that specific combinations of the 8 drugs tested exhibited strong synergy against ZEBOV trVLP, which were confirmed with fully infectious ZEBOV-GFP in BSL4. FPV showed synergy with all other drugs tested, suggesting recombinant IFNs, TOR or nucleoside analogs could be used with FPV to reduce its monotherapy dose and maintain the same level of inhibition. This is in line with FPV combinations with ribavirin that synergistically inhibit other RNA viral infections, such as (-)ssRNA Lassa virus, Pichinde virus and RVFV, and (+)ssRNA Junin arenavirus [435, 436, 530]. Moreover, synergistic two drug combinations, such as IFN-β + 3TC or IFN-β + AZT, were predictive of the synergy when testing the three drugs together (IFN-β + 3TC + AZT). Of combinations only comprising nucleoside/nucleotide analogues, FPV + TFV showed the highest
synergy. This result was predicted *in silico* from their combined interaction energy displacing NTPs at the putative nucleotide binding motif of RdRP L, with FPV binding from the “left” and TFV binding from the “right” of the polymerase nucleotide binding pocket (see Figure 5.2 for binding of TFV + FPV outcompeting NTP binding, originally published in [456]). In addition, the *in vitro* trVLP model in chapter 4 permitted rapid testing of multiple drug combinations, with hits inhibiting luciferase activity as high as 97.3% and with no appreciable affect on cell viability. This data allowed for 14 combinations to then be prioritized for BSL4 testing at the National Microbiology Laboratory in Winnipeg. As in ZEBOV trVLP infection, the combinations of IFN-β + 3TC or IFN-β + 3TC + AZT showed the strongest drug synergy against ZEBOV-GFP *in vitro*. Moreover, the consistency in which low doses of recombinant IFN-β inhibited ZEBOV alone or in combination added to the strong preclinical evidence supporting this treatment to be considered in a phase II ZEBOV clinical trial [22, 31]. On March 26th 2015, a phase II IFN-β-1a proof-of-concept, historically controlled, single-arm trial started in the western town of Coyah, Guinea, and completed by June 12th 2015 [22]. While the results in chapter 4 are but one of many sources of data that contributed to the planning of this trial, it is an example of real-world impact this thesis has already made.

Presently, there are no active cases of acute EVD reported by the WHO in West Africa [5]. Yet from May to June 2017, a small outbreak of EVD (5 confirmed cases, 3 probable cases) occurred in the North-East province of Likati in the Democratic Republic of the Congo [604]. While the WHO is confident this new outbreak will be contained, this should not engender research complacency between larger EBOV outbreaks. On the contrary, this time provides an opportunity for clinicians and scientists to produce rigorous and sound preclinical evidence that better inform policy makers, who may again priorotize EBOV clinical trials during an active EBOV outbreak. In our lab, future work has begun on screening antimalarial derivatives of quinoline, to determine antiviral activity in the ZEBOV trVLP model of infection in 293T cells. Moreover, earlier work on SFK inhibitors that inhibit trVLP replication has been
Fig. 5.2: Model of FPV-RTP and TFV-DP binding the RdRP L nucleotide pocket of ZEBOV. Preferential binding of favipiravir-RTP (red) to the “left” side and tenofovir-DP (orange) to the “right” side of the nucleotide binding motif (yellow). The total interaction energy of both nucleotide analogues with the polymerase is significantly lower than each NTP (green), displacing them to locations with decreased affinity. (A) ATP. (B) CTP. (C) GTP. (D) UTP [456].
reinitiated. This investigation of host tyrosine kinases will likely be a promising new area of research, as 53 host-protein interactions during ZEBOV infection of VeroE6 cells have recently been described, and little is known of the role of NRTK signaling during ZEBOV replication [357]. Only two such studies have been published [605, 606]. The first showed that ABL1 phosphorylation of VP40 matrix protein at Tyr13 was essential for budding of newly assembled ZEBOV virions, by employing siRNA or a specific inhibitor (nilotinib) targeting ABL1 activity [605]. The second publication also used FDA-approved kinase inhibitors (sunitinib and erlotinib) to inhibit ZEBOV entry [606], providing additional rationale to explore SFK signaling and the repurposing of kinase inhibitors in future ZEBOV lifecycle research.
5.2 Conclusions and Broader Significance

HIV-1

In summary, this work has specifically shown that c-SRC and PTK2B non-receptor tyrosine kinases are key mediators of early HIV-1 replication in various T-cell lines and in activated primary CD4+ T-cells from healthy donors. Using multiple experimental methods (including tool-drug inhibitors, adenovirus vectors overexpressing DN c-SRC mutant, and targeted siRNA knockdown) and testing of various strains of HIV-1 viruses (X4- or R5-tropic, fully infectious or nef-deleted luciferase reporter viruses, VSV-G pseudo-enveloped viruses, laboratory strains and primary clinical isolates), it was determined that reducing the activity or expression of c-SRC consistently increased viral infection in transformed T-cell lines, but reduced viral infection in primary CD4+ T-cells. Results with primary CD4+ T-cells were taken as the more physiological relevant results and further examined. Inhibition of viral infection of CD4+ T-cells was associated with no change in RT activity, increased early and late reverse transcripts, reduced proviral integration, and higher accumulation of 2-LTR circles, suggesting two novel mechanisms by which this kinase may be altering intracellular HIV-1 replication: 1) At the level of the pre-integration complex formation in the cytosol, and 2) During PIC nuclear translocation and viral integration. Similar results were discovered for the c-SRC binding partner PTK2B. However reduced PTK2B kinase expression only appeared to decrease HIV-1 infection when R5 viruses were tested. These findings offer the potential for better characterization of the PIC and its associated host proteins, as arresting the virus at this stage from c-SRC siRNA knockdown could allow for better isolation of this elusive protein/cDNA complex that is hard to characterize in primary CD4+ T-cells [138, 607, 608].

The dependency of early HIV-1 infection on c-SRC signaling was a novel and complex finding. This was unlike other SFKs FYN and LCK, which transmit signals as HIV-1 binds and fuses at the plasma membrane, interact with viral Nef (LCK), change the recycling of surface proteins at the trans-Golgi network (LCK), phosphorylate APOBEC3G (FYN) or facilitate viral egress (LCK) [208, 217, 218, 223, 227, 238]. The work presented here strongly supports that c-SRC has a unique cytosolic and nuclear role in the cell biology of HIV-1 infection of CD4+ T-cells. It
demonstrates that c-SRC regulates HIV-1 infection at multiple stages post-entry, reminiscent of the integrase inhibitor raltegravir with respect to the nuclear effects of c-SRC [565, 566]. Furthermore, this research shows that four specific inhibitors of c-SRC (dasatinib, saracatinib, KX2-391 and SRC Inhibitor-1), each with different off-target activity, can significantly reduce HIV-1 infection of CD4+ T-cells. These four inhibitors, FDA-approved or under clinical evaluation as potential cancer treatments [553, 561, 609], showed excellent cell viability data at doses that significantly inhibit HIV-1 replication ex vivo. These results lay the foundation for further preclinical testing of dasatinib, alone or concurrent with cART treatment, in a humanized mouse model of HIV-1 infection.

The findings in this work make a significant advance in the fields of HIV-1 cell signalling and novel HIV-1 treatment strategies, by demonstrating the roles of two non-receptor tyrosine kinases during early HIV-1 entry of CD4+ T-cells, and evaluating kinase inhibitors that can specifically inhibit one of these two targets. The fundamental roles of host kinases in the HIV-1 lifecycle are increasing being recognized, allowing for rapid discovery of kinase inhibitors as new potential therapies of HIV-1 infection [146, 156, 160, 610]. Small-molecule kinase inhibitors that can be repurposed for new indications in virology offer many significant advantages [169, 170]. They often have low toxicity profiles, and broad tissue distribution. In vitro studies suggests they may offer unique ways to inhibit HIV-1 replication in viral sanctuaries [560, 611, 612], potentially altering the dynamics of the latent viral reservoir. They can also be paired with latency reversal agents to prevent unwanted inflammation during “shock and kill” methods [173]. Moreover, kinase inhibitors have the potential for ameliorating underlining inflammation and neural cognitive dysfunction that occur during HIV-1 infection [173, 613], which are not addressed adequately with current cART regimens. Kinase inhibitors could also reduce the risk of cancers in HIV-1 patients, as demonstrated by their ability to treat KS or CML without causing additional complications, extending the life expectancy of these HIV-1 patients [176, 600]. Additionally, targeting host kinases essential for early intracellular infection may pose higher barriers towards selecting drug-resistant mutations, as they would not directly inhibit viral proteins [135, 159, 539]. Thus, tyrosine kinase inhibitors could be a useful part of a multidrug regimen for those living with HIV-1. There is a strong need to design not
only intensifying therapies, but therapies that improve immune function in HIV-1 patients, such as re-establishing CD4+ T-cell populations at gut mucosa [80]. The clinical application of SRC-family kinase inhibitors of known specificity, biodistribution, pharmacokinetics, toxicity profile and ease of administration, suggest these drugs could be repositioned as novel inhibitors of HIV-1 infection, and should be explored further in small animal models of HIV-1 disease progression.

ZEBOV

The 2014-16 outbreak of ZEBOV in West Africa challenged global healthcare workers and virology researchers from different backgrounds to come together and quickly assess which preclinical drugs, treatments and vaccines should be prioritized for emergency evaluation in phase II/III ZEBOV clinical trials. Many new molecules were discovered to have \textit{in vitro} antiviral activity [586], or improved the survival in animal models when treated after an otherwise lethal ZEBOV exposure [6, 25]. The phase II/III ZEBOV clinical trials were the first of their kind in an outbreak setting during a humanitarian crisis, which led to many lessons for future trials, such as: ethical considerations of consent and the use of placebos; standardization of intensive supportive care; the fostering of local, regional and global partnerships; establishing multiple treatment sites as the spread of disease rapidly shifted; and new trial formats such as the cluster-randomized ring design [16, 25, 26]. In particular, multiple approaches from HIV-1 drug research, spanning from preclinical drug screening to treating patients who suffer not only from EVD, but associated disease stigmatization, were very relevant in Liberia, Guinea and Sierra Leone during the ZEBOV outbreak.

The creation of a sophisticated \textit{in vitro} lifecycle model of ZEBOV in 2014 [534] allowed for rapid assessment of 8 different drugs from three drug classes, including toremifene, nucleoside/nucleotide analogues, and recombinant interferons. This thesis made significant contributions by comparing and contrasting each of these compounds, testing various time-points, doses, and 84 unique combinations, while assessing cell viability and antiviral activity against a ZEBOV trVLP luciferase reporter virus. It was demonstrated for the first time that safe and well tolerated nucleoside analogues 3TC, AZT and TFV, often used to treat HIV-1 or HBV infection [408, 410], can inhibit ZEBOV trVLP replication \textit{in vitro}. This was confirmed with
qRT-PCR measurements of ZEBOV vRNA and cRNA/mRNA synthesis 2 days post-infection. The antiviral activity of 3TC, AZT and TFV were comparable or lower than the IC$_{50}$ of nucleoside analogues fast-tracked for phase II trials in Guinea and Liberia: FPV and the metabolite of BCV (CDV) [18, 24]. While neither FPV nor BCV demonstrated significant therapeutic benefit in these clinical trials, lessons were learned of unwanted side effects from BCV administration [24], and lower than anticipated plasma levels from FPV monotherapy [18]. Retrospective analysis of compassionate FPV use suggest this drug should continue to be developed for experimental treatment of EVD in a future EBOV outbreak, in addition to intensive supportive care [445].

Testing 5 nucleoside/nucleotide analogs in the trVLP replication model confirmed many of the *in silico* predictions of how these drugs might dock to the nucleotide binding motif of RdRP L and outcompete NTP substrates [456]. TFV was predicted to have the strongest interaction energy with the enzyme [456], and it was confirmed to have the lowest IC$_{50}$ of the 5 nucleoside analogs tested. Moreover, simulations of two nucleoside analogs flanking the nucleotide binding motif from the “left” and “right” predicted strong synergy when combining TFV with FPV [456], a drug combination that experimentally was confirmed to be highly synergistic *in vitro*. In addition, testing of two-drug combinations rationally predicted synergistic three drug combinations, adding more strength to the methodology used [533]. Lastly, confirmation of antiviral activity of the 14 most synergistic drug combinations occurred with fully infectious ZEBOV expressing a GFP reporter, demonstrating multiple efficacious combinations that included FPV or IFN-β. Thus both of these drugs, which were evaluated in phase II ZEBOV clinical trials as monotherapies [18, 22], could very well be improved by combinations with other nucleoside analogues. These hypotheses should be investigated further in small animal or non-human primate models of lethal ZEBOV challenge.

Combinations of monoclonal antibodies and siRNAs were behind the early success of ZMapp and TKM-Ebola; however, they had shortcomings in drug production or treating people with EVD in controlled clinical trials [27, 404]. Combinations with nucleoside analogues are now on the leading edge of EBOV drug discovery, as ZMapp is actively being studied in combination with either FPV or recombinant IFN-β. Thus, the nucleoside analogues 3TC, AZT and TFV
could fill the gap where monotherapy has so far failed to demonstrate therapeutic benefit, by increasing the efficacy of other administered treatments as combination regimens. Moreover, nucleoside analogues may have the additional advantage of exploiting the spontaneous mutation rate of ZEBOV [614]. While the frequency of ZEBOV mutations are high, as other RNA viruses, they are not represented in the genetic diversity of viable strains, suggesting nucleoside analogues that increase the error rate of RdRP cause harmful mutagenesis, reducing the fitness of ZEBOV during infection [614]. Nucleoside analogs are also on the WHO list of essential medicines, and can be deployed in resource limited settings, often without need of cold storage [574]. Taken together, the findings presented in this thesis demonstrate the value of an in vitro BSL2 trVLP screening system prior to BSL4 ZEBOV testing, allowing for direct comparisons of 8 compounds with suggested or known antiviral activity against ZEBOV replication [29, 31]. This work also contributes to the growing body of evidence advocating for combination therapy as standard treatment of RNA viruses and retroviruses, to improve efficacy and theoretically reduce the risk of drug resistance [456, 523, 530, 532, 615]. In future studies, this ZEBOV trVLP model will continue to allow rapid assessment of new compounds of interest, such as SFK inhibitors and quinoline derivatives, to directly compare their antiviral activity with leading compounds in ongoing or planned ZEBOV clinical trials.

As of June 2016, there are approximately 17,300 Ebola survivors in West Africa, the largest such Ebola cohort ever recorded [5]. The management of chronic disease that is becoming increasingly recognized as post-Ebola syndrome, is under active investigation [6, 450]. Moreover the mechanisms of viral persistence, and the risk of ZEBOV reemergence and shedding, are also being actively studied [357, 360]. Ebola survivors with persistent symptoms and low-level viral replication in immune-privileged organs may benefit from antiviral drugs with appropriate permeability, such as the nucleoside analog GS-5734 [417]. ZEBOV virions have been found in the semen of male Ebola survivors, which can cause acute EVD if transmitted to their partner, suggesting ZEBOV virus transmission can be similar to other sexually transmitted viruses [360]. Hence clinical trials testing the efficacy of nucleoside analogues to reduce the shedding of virions in the semen of male Ebola survivors are currently underway: a phase II FPV trial in Guinea and a phase II GS-5734 trial in Liberia [419].
The successful rVSV-ZEBOV-GP cluster-randomized ring vaccine trial in Guinea, which protected 100% of those immediately vaccinated (N = 2,119) compared with those receiving delayed vaccination (N = 2,041) [26], may provide protection in future ZEBOV outbreaks for health care workers and quarantined contacts of EVD patients. Until the vaccine demonstrates durable protection and broad neutralization of the five *Ebolavirus* species (ZEBOV, SUDV, BDBV, RESTV and TAFV) a future outbreak emerging from a new EBOV strain may be resistant to the current rVSV-ZEBOV-GP formulation. Thus, the rVSV-ZEBOV-GP vaccine is one of many components in the EBOV toolkit needed to reduce the spread of future infections. Government cooperation, effective border screening of EVD symptoms, robust healthcare infrastructure, extensive training of healthcare personnel, proper Personal Protective Equipment (PPE), rapid onsite EBOV antigen testing, contact tracing, community engagement, public health education, and access to the basic necessities of life, all contribute to the epidemiological spread and control of an EBOV epidemic. Thus, an effective treatment or combination of treatments could have far reaching consequences in the transmission of EBOV in addition to immediate patient care. An effective treatment could provide psychological reassurance that improves trust between healthcare staff and ill patients, encouraging people to seek treatment at an ETC and reduce EBOV spread among family caregivers at home. An emergent EBOV zoonose may also show higher lethality compared to the ZEBOV-Makona and ZEBOV-Kikwit strains of the most recent EBOV outbreak. For all of these reasons, future research into effective nucleoside analog drug combinations should continue to proceed in preclinical *in vivo* models of EBOV infection, until there is an effective treatment for acute and chronic EBOV infection.


Fenouillet E, Jones IM. The glycosylation of human immunodeficiency virus type 1 transmembrane glycoprotein (gp41) is important for the efficient intracellular transport of the envelope precursor gp160. *J Gen Virol* 1995; 76:1509-1514.


311. Chong YP, Mulhern TD, Cheng HC. C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK)--endogenous negative regulators of Src-family protein kinases. *Growth Factors* 2005; 23:233-244.


573. Phanuphak N, Ananworanich J, Teeratakulpisarn N, *et al.* A 72-week randomized study of the safety and efficacy of a stavudine to zidovudine switch at 24 weeks compared to zidovudine or tenofovir disoproxil fumarate when given with lamivudine and nevirapine. *Antivir Ther* 2012; 17:1521-1531.


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