Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Young-Jae Cho April 13, 2021

Exploring the potential of organic compounds as antiviral agents against HIV-1

by

Young-Jae Cho

Baek Kim
Adviser

Biology

Baek Kim

Adviser

Patrick William Cafferty

Committee Member

John Lindo

Committee Member

2021

Exploring the potential of organic compounds as antiviral agents against HIV-1

By

Young-Jae Cho

Baek Kim

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Biology

2021

Abstract

Exploring the potential of organic compounds as antiviral agents against HIV-1
By Young-Jae Cho

Human immunodeficiency virus type 1 (HIV-1) is a member of the *Lentivirus* genus classified under the *Retroviridae* family. It was initially suggested to have been introduced into the human population via zoonotic transmission of primate lentiviruses. HIV-1 targets CD4+ T-cells, dendritic cells, and macrophages, which, if left untreated, progresses into acquired immunodeficiency syndrome (AIDS). Currently, HIV-1 viral loads are controlled using highly active antiretroviral therapy (HAART), a combination of three drugs of two or more differing origins. HAART has been crucial in suppressing HIV-1 to levels that are undetectable in some treated patients. However, an increase in the use of HAART to control HIV-1 infection, mutations in the viral enzymes due to replication errors, and the ability of the virus to rapidly replicate within the host have facilitated the emergence of viral strains that are resistant to drugs currently used in HAART. Therefore, there is a need to develop novel anti-HIV-1 agents to combat emerging resistant strains of HIV-1. In this study, we aim to identify organic compounds and mixtures of organic compounds with great inhibitory potencies against HIV-1 while exhibiting low cytotoxicity in a human T-cell line (CEMx174). A total of 35 organic compounds and mixtures were screened for cytotoxicity in an MTS assay using CEMx174 cells and screened for HIV-1 antiviral potencies using CEMx174 cells infected with HIV-1-D3-GFP vector. Out of the 35 compounds and mixtures screened, only one mixture, BK 91, exhibited both high cell viability relative to cell control (102.7%) and significant reduction in percent GFP expression (55.65±1.10, $p<0.05$) relative to virus control, which indicated significant viral suppression. Subsequent in-depth analysis of BK 91 determined the mixture's CC_{50} (≈57.47 μg/mL) and EC_{50} range (between 6.5 μg/mL and 8.5 μg/mL). Further study is needed to isolate and analyze the individual compounds that comprise BK 91 to determine which components contribute to its anti-HIV-1 activity, as well as comparing the characteristics of BK 91 to HAART drugs currently used.

Exploring the potential of organic compounds as antiviral agents against HIV-1

By

Young-Jae Cho

Baek Kim

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Biology

2021

Acknowledgements

I would like to thank my research adviser, Dr. Baek Kim, my direct mentor, Adrian Oo, Ph.D., and the rest of the members of the Baek Kim Laboratory for their tremendous support and guidance throughout the entire duration of this study. I would also like to thank Dr. Jong-Hyun Cho of Dong-a University's Laboratory of Antiviral Drug Discovery in Busan, South Korea, for supplying the compounds and mixtures screened in this study. Finally, I would like to thank my committee members Dr. Patrick William Cafferty and Dr. John Lindo for providing crucial feedback and support during this study. Research reported in study was supported by the National Institutes of Health under award numbers 5R01AI136581 and 8R01AI150451.

Table of Contents

Introduction

Origin and Global Impact of HIV-1

Originating in west equatorial Africa in the late 20th century, the first cases of human immunodeficiency virus type 1 (HIV-1), can be traced back to a zoonotic transmission of simian immunodeficiency virus (SIV) to humans, with early strains of HIV-1 having nearly identical genome structure to SIV*cpz*, a chimpanzee strain of SIV (Hahn et al., 2000). HIV-1 consists of three groups, M, N, and O, which are believed to correspond to three separate zoonotic transmission events (Taylor et al., 2008). Phylogenetic analysis indicates that the Group M and Group N originate from a strain of SIV from chimpanzees, while the Group O originated from a strain of SIV from gorillas (Van Heuverswyn et al., 2006). Group M is the dominant group of HIV-1 and is further split into nine subtypes: A, B, C, D, F, G, H, J, and K (Hemelaar et al., 2006).

Since its emergence in human populations, HIV-1 has had a devastating impact on human populations around the world, with The Joint United Nations Programme on HIV and AIDS (UNAIDS) reports that in 2019, an estimated 38.0 million people were living with HIV, with an estimated 1.7 of the cases being those who have been newly infected with the virus (UNAIDS, 2020). From the start of the HIV epidemic to 2019, 75.7 million people has become infected with HIV, and 32.7 million people have died from AIDS-related illness (UNAIDS, 2020). While this data includes HIV-2, an HIV first discovered in West Africa, most HIV-2 cases are from West Africa and make only a small fraction of total HIV infections worldwide (de Silva et al., 2008). Regions of the world severely impacted by HIV include eastern and southern Africa, Asia and the Pacific, and western and central Africa, which had an estimated 20.7 million people, 5.8 million people, and 4.9 million people living with HIV in 2019, respectively (UNAIDS, 2020).

Clinical Definition of HIV/AIDS

The major targets of HIV-1 are cells that express high densities of CD4 receptors, such as CD4+ T-lymphocytes, dendritic cells, and macrophages (Fanales-Belasio et al., 2010). Individuals initially infected by HIV-1 enters an acute infection phase, where individuals can experience symptoms such as fever, maculopapular rashes, headaches, pharyngitis, weight-loss, and oral or genital ulcers, which can last for less than 14 days, but can go on for more than 10 weeks (Kahn and Walker, 1998). This period of acute infection is characterized by a sudden drop in the host's CD4+ cell count, which gradually increases until the asymptomatic phase of the infection (Fanales-Belasio et al., 2010). During the asymptomatic or clinical latency period, the host's immune system continues to become impaired as the CD4+ lymphocyte count continues to decrease due to the virus (Fanales-Belasio et al., 2010). As the host's CD4 cell count gradually declines, they may experience diseases caused by opportunistic infections such as thrush, tuberculosis, salmonella, and toxoplasmosis (Gazzard, 1995).

Without treatment, this continued degradation of the host's immune system can lead to acquired immunodeficiency syndrome (AIDS), often described as the host having a CD4+ lymphocyte count lower than 200 cells/mm^3 (Battistini Garcia and Guzman, 2020; Brooks et al., 2009). As a result of the low CD4 cell count, this stage of HIV infection often includes life-threatening opportunistic infections caused by pathogens such as *Herpes zoster*, *Candida albicans*, *Cytomegalovirus*, and intestinal tract parasites (*Criptosporidium*, *Giardia* species, *Isospora belli*) (Fanales-Belasio et al., 2010). Symptoms experienced during the AIDS stage include rapid weight loss, widespread swelling of the lymph nodes, fever, and issues in the respiratory and gastrointestinal systems, such as a dry cough or diarrhea (Fanales-Belasio et al.,

2010). The average time to death from initial HIV infection to death by AIDS is approximately 11 years (Fanales-Belasio et al., 2010; Todd et al., 2007).

HIV-1 Structure, Transmission, and Replication Cycle

HIV-1 particles are approximately 100 nm in diameter and are surrounded by a lipoprotein-rich membrane whose exterior is embedded with glycoprotein heterodimer complexes composed of trimers of non-covalently bonded envelope glycoproteins gp120 and gp41 (Fanales-Belasio et al., 2010). Gp41 is a transmembrane protein, while gp120 is expressed only on the exterior surface of the virus (Fanales-Belasio et al., 2010). Structures located in the interior of the virus membrane include the virus matrix, which is composed of the structural protein p17 attached to the viral membrane, and the viral capsid, which contains the viral genome and reverse transcriptase enzymes (Fanales-Belasio et al., 2010).

The HIV-1 genome, which is composed of two identical, positive-sense, single-stranded RNA, contains three key structural genes: *Gag*, *Pol*, and *Env* (Fanales-Belasio et al., 2010; German Advisory Committee Blood, 2016). The *Gag* gene encodes for a precursor polyprotein which, when processed, yields structural proteins of the cone-shaped virus core (p24, p7, and p6) and matrix (p17) (Fanales-Belasio et al., 2010). The *Pol* gene encodes for reverse transcriptase that converts viral RNA to DNA, integrase required to incorporate viral DNA into the host genome, and protease that cleaves precursors of Gag and Pol proteins. The *Env* gene encodes for the viral envelope glycoproteins gp120 and gp41, which are used to identify surface cell receptors on potential host cells as well as in cell entry (Gelderblom et al., 1989). Additionally, the *Nef* gene, responsible for the expression of the accessory protein Nef, is responsible for the activation of infected T-cells, the downregulation of key surface cell receptors on cells of the

immune system such as MHC-1 and MHC-2 on antigen presenting and target cells, and CD4 and CD28 receptors on CD4+ cells (Das and Jameel, 2005). The downregulation of CD4 receptors on infected cells allows for virus budding in the late stages of the HIV-1 replication cycle (Fanales-Belasio et al., 2010). This allows for the host to be in a state of persistent HIV-1 infection (Das and Jameel, 2005). Another accessory gene, *Tat*, encodes for the Tat protein, which enhances the transcription and replication of HIV-1 genes (Debaisieux et al., 2012; Fanales-Belasio et al., 2010).

HIV is mainly transmitted through direct exposure to infected blood or other infected bodily fluids such as semen, vaginal fluids, pre-seminal fluid, rectal fluids, and breast milk. HIV is known for having poor survivability outside of the bloodstream or lymphatic tissue, and is inactivated by common detergents and disinfectants, making transmission of the virus from outside surfaces unlikely (Fanales-Belasio et al., 2010). Therefore, in order to be infected with HIV, the infected fluid must make direct contact with damaged skin, such as through the use needles, or through abrasions caused on mucosal tissues, such as during sexual intercourse (Fanales-Belasio et al., 2010).

Once inside the host, the HIV-1 replication cycle first involves the fusion of the viral particle to a cell surface receptor, usually CD4 (Fanales-Belasio et al., 2010). The virus envelope, upon binding, undergoes a structural change that ends with the envelope fusing with the host membrane and allows the viral capsid core to enter the host cell and subsequently dissociate in the cytoplasm (Fanales-Belasio et al., 2010). It is then that reverse transcriptase converts viral RNA into DNA, which is then integrated into the host DNA by viral integrase (Fanales-Belasio et al., 2010). Integration into the host genome allows more copies of viral RNA and proteins to be made using the host's machinery, which migrate to the surface of the cell,

eventually forming an immature HIV-1 particle (Fanales-Belasio et al., 2010). Precursor molecules in the immature viral particles are cleaved by HIV-1 protease, and a new viral particle eventually buds off of the host cell's membrane and is released, ready to infect other cells (Fanales-Belasio et al., 2010).

Highly Active Antiretroviral Therapy (HAART)

Currently, there is no known cure for HIV-1. However, HIV-1 viral loads are controlled through highly active antiretroviral therapy (HAART), which involves a custom combination of two or more drugs of different classes that target different steps of the HIV-1 replication cycle (Pau and George, 2014). In some cases, HAART allows for enhanced suppression of viral replication to lower viral levels below the limit of detection (Pau and George, 2014). Here, we will cover general functions and notable and/or novel compounds of four main classes of antiviral compounds used in HAART: protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and integrase inhibitors.

Protease Inhibitors

Protease inhibitors (PIs) act towards the end of the HIV replication cycle, binding to HIV protease's active site and preventing viral protease from cleaving the precursor molecules that give rise to infectious HIV particles, resulting in incomplete viral particles incapable of infecting other host cells (Pau and George, 2014). One notable feature of PIs is their high genetic barrier, which makes it difficult for viral particles to gain resistance to the compounds without multiple mutations (Spagnuolo et al., 2018). Common side effects of protease inhibitors include metabolic syndromes such as dyslipidemia, hyperglycemia, insulin resistance, and lipodystrophy, as well as

cardiovascular and cerebrovascular diseases (Bozzette et al., 2003; Pau and George, 2014). In 2015, there were nine PIs approved by the FDA: saquinavir, indinavir, ritonavir, nelfinavir, fosamprenavir, lopinavir, atazanavir, tipranavir, and darunavir (Pau and George, 2014). These compounds share a similar structure as well as a similar mechanism of binding to viral protease, as they are designed to mimic the substrate transition state (Lv et al., 2015). The inhibitors contain a hydroxyl group, which forms hydrogen bonds with the carboxyl group of the Asp 25 and Asp 25' residues of the protease's active site (Lv et al., 2015). Of the listed protease inhibitors, ritonavir is used alongside all PIs in low doses (100 to 400 mg per day) as a pharmacokinetic enhancer, inhibiting the metabolism of active PIs, resulting in both higher drug exposure and plasma concentration inside the body, permitting lower dose and dose frequency (Kempf et al., 1997). This is due to ritonavir's ability to inhibit the cytochrome P450 3A4 (CYP3A4) enzyme, which is present in both the liver and the intestinal tract (Hull and Montaner, 2011). For the following paragraphs, we will cover the most widely used PI, darunavir, as well as a study that produced two novel compounds that may prove more effective.

As the most recent PI to be approved by the FDA, darunavir (DRV) is considered to have one of, if not the highest genetic barriers to HIV-1, is recommended as a first-line therapeutic, and is the most extensively used PI (Aoki et al., 2018). DRV, besides inhibiting the active site of HIV-1 protease, also prevents protease dimerization of the protease's subunits (Hayashi et al., 2014). DMV is similar in structure to amprenavir, only the P2 group bis-tetrahydrofuran replaces the tetrahydrofuran group found on amprenavir, allowing for more hydrogen bonds with Asp 29 residues on the protease (Lv et al., 2015). It is usually co-administered with a low dose of ritonavir, as the metabolism of DRV depends on the cytochrome P450 3A4 enzyme (Spagnuolo et al., 2018). When boosted with 100 mg of ritonavir, DRV reached peak serum concentration

within 2.5 to 4 hours and had a 14-fold increase in systemic exposure compared to when administered alone (Spagnuolo et al., 2018). Common side effects for this treatment includes rash, diarrhea, nasopharyngitis, and nausea (Lv et al., 2015). Metabolic complications associated with PIs, such as dyslipidemia and hyperglycemia, were found to have occurred in a smaller percentage of patients taking DRV and ritonavir compared to patients taking a lopinavir and ritonavir treatment (Orkin et al., 2013). However, although DRV has a high genetic barrier, mutant HIV-1 strains resistant to the compound have been observed both *in vivo* and *in vitro* (De Meyer et al., 2009; Koh et al., 2010).

In a study conducted by Hattori *et al.* published in 2019, two new protease inhibitors, GRL-001-15 and GRL-003-15, were demonstrated to have higher antiviral properties than DRV (Hattori et al., 2019). These two compounds both contain P2-crown-THF (P2-Crn-THF) and P2'-cyclopropyl-aminobenzothiazole moieties (P2'-Cp-Abt), and, on the P1-benzene, contains a fluorine atom at the *meta* position for GRL-001-15 and *beta* position for GRL-003-15. With favorable cytotoxicity concentrations, the compound's selectivity indexes were both much higher than that of DRV, 666,666 and 220,000 for GRL-001-15 and GRL-003-15, respectively compared to 45,121 for DRV, indicating both had greater effectivity and were safer to use *in vivo*. When tested against laboratory-selected and clinically isolated strains of HIV-1 that were immune to saquinavir, amprenavir, lopinavir, indinavir, nelfinavir, atazanavir, and tipranavir, both compounds had much greater antiviral activity than DRV, which only had a moderate effect in combating the strains. Additionally, the compounds bound tighter to HIV-1 protease and slightly higher lipophilicity indexes than DRV. The tighter binding and the higher antiviral activity of both compounds can be attributed to the fact that the P2-Crn-THF and P2'-Cp-Abt residues are noticeably larger than their counterparts on DRV, and they form 11 direct and 2

water-mediated hydrogen bonds and favorable van der Waals interactions with wild-type protease. The para-fluoride atom of GRL-003-15 formed a halogen bond with Pro81 of wild-type protease, while the meta-fluoride atom of GRL-001-15 formed two halogen bonds, with Gly49 and Ile50, with the flap region of wild-type protease. This allowed GRL-001-15 to be a better candidate for a novel antiviral agent, as tests with laboratory-selected PI-resistant HIV-1 strains and wild-type HIV-1 strains showed reduced antiviral resistance for GRL-003-15 (Hattori et al., 2019).

Nucleoside Reverse Transcriptase Inhibitors

Nucleoside reverse transcriptase inhibitors (NRTI) were the first class of antiviral agents for HIV-1 approved by the FDA, with zidovudine in 1987 (Fischl et al., 1987). Since then, NRTIs have been an integral part of HAART, with treatments consisting of 2-NRTI as a backbone along with other antiviral agents (Pau and George, 2014). As their name suggests, NRTIs are competitive inhibitors that inhibit the conversion of HIV-1 RNA to double-stranded DNA in the host cell's cytoplasm, preventing the incorporation of viral RNA into the host genome (Pau and George, 2014). These compounds act as nucleotide base analogs by phosphorylating intracellularly and subsequently incorporating into the replicating viral DNA (Pau and George, 2014). NRTIs are effective chain-terminators of replicating double-stranded DNA after incorporating due to replacing the hydroxyl group required for the addition of new nucleotides with a non-reactive group (Sohl et al., 2012). However, NRTIs, especially older NRTIs, have been shown to have critical toxicity, especially on human mitochondrial DNA in certain tissues, leading to increased risk of conditions such as cardiomyopathy (Birkus et al., 2002; Kohler et al., 2008). Other side effects associated with this type of agent have been lipoatrophy, lactic acidosis,

hepatic steatosis, bone marrow toxicity, peripheral neuropathy, and myopathy (Pau and George, 2014). Of the NRTIs used today, tenofovir and abacavir are the most widely utilized in treatments. Here, we will discuss tenofovir as well as novel nano-NRTIs.

Tenofovir, also known tenofovir disoproxil fumarate (TDF), combined with emtricitabine or lamivudine, with the addition of a third antiviral agent, has been shown to have a potent effect in inhibiting HIV-1 as well as hepatitis B (HBV) (Pau and George, 2014). However, the use of TDF has been associated with nephrotoxicity, which may worsen with the use of a PI (Kalayjian et al., 2012). Therefore, alternative tenofovir compounds with a reduction of nephrotoxicity have been explored, with one notable compound being tenofovir alafenamide (TAF). TAF, approved in 2015 in the United States and Europe, has been shown to have a 30-fold lower dose (10 mg) than TDF (300 mg) (De Clercq, 2016). Compared to TDF, TAF regiments consisting of TAF, emtricitabine, and a third agent have been shown to have lower kidney toxicity and bone mineral density changes in the spine and hip (De Clercq, 2016).

Another study conducted by Gerson *et al.* published in 2014 addressed the issue of low efficiency of NRTI concentrations in the central nervous system with nano-NRTIs (Gerson et al., 2014). NRTIs, due to being both neurotoxic and having low accumulation in the CNS due to low permeability with the blood-brain barrier, has low efficiency in removing HIV-1 (Makarov et al., 2010). By generating two nano-NRTIs (NG1 and NG2) decorated with a peptide (AP) binding brain-specific apolipoprotein E receptor, compared to NRTIs, lower levels of apoptosis and a reduction in the generation of reactive oxygen species was observed, indicating lower risk of cell death. Additionally, nano-NRTIs coated with AP were able to be more efficiently accumulate in the CNS compared to NRTIs, resulting in a 10-fold suppression of retroviral activity in the CNS (Gerson et al., 2014).

Non-nucleoside Reverse Transcriptase Inhibitors

Non-nucleoside reverse transcriptase inhibitors (NNRTI) inhibit viral reverse transcription during its replication cycle, much like their nucleoside counterparts. However, unlike NRTIs, NNRTIs do not need to undergo phosphorylation intracellularly to inhibit retroviral activity and are noncompetitive inhibitors to reverse transcriptase, changing the conformation of the protease upon binding to a hydrophobic site near the active site of reverse transcriptase (Pau and George, 2014). As of 2019, there are six NNRTIs in use for treatment: nevirapine, etravirine, elsulfavirine, efavirenz, rilpivirine, and doravirine (Wang et al., 2019). All NNRTIs are metabolized by cytochrome P450 enzymes (Pau and George, 2014). While used as antiviral therapy due to their specificity and antiviral potency, they exhibit side effects such as rash, hyperlipidemia, dizziness, QT interval prolongation, hepatotoxicity, and even depression (Pau and George, 2014). Additionally, mutations in HIV-1 strains have limited use of both first- and second-generation of NNRTIs (Beyrer and Pozniak, 2017; Lehman et al., 2012). Here we will cover etravirine as well as novel fluorine-substituted diarylpyrimidine derivatives.

Out of all the NNRTIs used for antiviral therapy, etravirine has the most effective genetic barrier, showing effectiveness against many strains resistant to other NNRTIs (Deeks and Keating, 2008). Etravirine is a diarylpyrimidine analogue that can adapt to mutations at the binding site on the reverse transcriptase, through conformational and positional changes at the binding site (Deeks and Keating, 2008). Additionally, etravirine enhances formation of inactive p66 homodimers as well as increased processing of gag and gag-pol proteins earlier in the HIV replication cycle, reducing the production of viral particles from treated cells (Figueiredo et al., 2006). Known side effects of etravirine includes rash, hyperglycemia, and high cholesterol (Pau

and George, 2014). For adults, etravirine is recommended for those who developed resistance to both efavirenz and nevirapine and recommended to be taken with meals (Pau and George, 2014).

On the topic of diarylpyrimidine NNRTIs, a study conducted by Kang *et al.* published in 2020 explored novel fluorine-substituted diarylpyrimidine derivatives as NNRTIs (Kang et al., 2020). The researchers had previously generated five fused-pyrimidine-bearing novel compounds, which demonstrated greater antiviral effects and resistance to HIV-1 mutation profiles, but showed inhibition of the hERG gene, possibly due to a piperidine-linked benzesulfonamide or benzamide motif present in the structure. This observation was not favorable, as inhibition of the hERG gene can cause tachyarrhythmia. In order to solve this issue, using molecular hybridization and bioisosterism, the researchers designed a series of fluorine-substituted diarylpyrimidines that avoided incorporation of the piperidine-linked benzesulfonamide/benzamide motif, while adding a fluorine atom and a trifluoromethyl group to form hydrogen bonding to make up for the abandoned motif. Of the generated compounds, 24b, was effective against both the wild-type HIV-1 strain as well as multiple NNRTI resistant HIV-1 strains. Further analysis revealed that this was due to a favorable position of a fluoride atom both on the molecule and inside the binding pocket on the reverse transcriptase as well as interaction of its nitrile group with the main-chain carbonyl group of the H235 residue in the binding pocket (Kang et al., 2020).

Integrase Inhibitors

In order to inhibit HIV-1 replication, main function of integrase inhibitors, is to block viral integrase from incorporating replicated double-stranded viral DNA generated by reverse

transcriptase into the host genome (Hajimahdi and Zarghi, 2016). They comprise a diverse class of compounds, including hydroxylated aromatics, diketo acids, naphthyridine carboxamides, pyrrolloquinolones, dihydroxypyrimidine carboxamides, azaindole hydrixamic acids, 2-hydroxyisoquinoline-1,3(2H,4H)-diones, 6,7-dihydroxy-1-oxoisoindolines, quinolone-3-carboxylic acids and carbamoyl pyridines (Hajimahdi and Zarghi, 2016). However, there are currently four FDA approved integrase inhibitors, specifically integrase strand transfer inhibitors (INSTIs) which include raltegravir, elvitegravir, dolutegravir, and bictegravir. These integrase inhibitors bind to the active site of HIV-1 integrase, which forms a complex with the viral DNA, impairing strand transfer (Pau and George, 2014). Another type of integrase inhibitor, allosteric integrase inhibitors (ALLINIs), binds to a site on the HIV-1 dimer that is recognized by the host cell transcription factor lens epithelium-derived growth factor (Poeschla, 2008). This has been shown to reduce integrase binding to LEDGF/p75, which has been shown as a docking site for the integrase; LEDGF/p75 both tethers HIV integrase to chromatin and protect the integrase from degradation (Poeschla, 2008). Additionally, ALLINIs were shown to promote abnormal integrase multimerization, resulting in failure of viral maturation leading to structurally defective and noninfectious viral particles (Jurado et al., 2013). In patients with no previous exposure to antiviral therapy, an INSTI along with 2-NRTIs is the recommended treatment (Pau and George, 2014). Here we will cover the first INSTI, raltegravir, as well as novel pyrazolopyrimidines as allosteric integrase inhibitors.

The first approved INSTI, raltegravir, has been shown to have potent antiviral activity against HIV-1, with a 95% inhibitory concentration in human T-lymphocyte cultures (31±20 nmol/L) in vitro (Hicks and Gulick, 2009). As raltegravir is metabolized mainly through glucuronidation, drugs that induce the enzyme UGT1A1 should not be taken while taking

raltegravir. For example, rifampin, an inducer of UGT1A1, has been shown to decrease raltegravir concentrations by 38% to 61% (Hicks and Gulick, 2009). Common side effects associated with raltegravir, though uncommon, includes skeletal muscle toxicity, proximal myopathy, creatine kinase elevation, and rhabdomyolysis (Lee et al., 2013; Pau and George, 2014).

In a study conducted by Li *et al.* published in 2020 demonstrates the potential for pyrazolopyrimidines as potent ALLINIs (Li et al., 2020). This study attempted to generate novel pyrazolopyrimidines that could successfully bind to the LEDGF/p75 binding pocket that would accommodate an integrase dimer using a computer-aided drug design (CADD) approach. Analysis of four diastereomers with HIV-1 integrase revealed that one, (*S*,*S*)-14, occupied the same binding pocket as LEDGF/p75. Further crystal-structure analysis revealed that substituents at the *meta*-position on the 2-phenyl group can extend better into the binding pocket, which the researchers believed would allow better binding affinity and antiviral activity. The most effective compound in the study, 29, demonstrated a half maximal effective concentration (EC_{50}) of 0.003 μM. Overall, three compounds, compounds 27 to 29, were shown to have favorable pharmacokinetic profiles with great oral bioavailability as well as favorable antiviral activity (Li et al., 2020).

HIV-1 Resistance to HAART

The increased use of antiretroviral drugs to treat HIV-1, mutations in targeted HIV-1 enzymes caused by errors in replication, and rapid replication of the virus in human hosts, have contributed to rapid selection of strains of HIV-1 that are resistant to HAART (Sarafianos et al., 2009; Shafer et al., 2000). In 2019, the World Health Organization (WHO) reports that globally,

25.4 million people out of 38.0 million people (67%) living with HIV received antiretroviral treatment (WHO, 2020). Additionally, up to 26% of people initiating antiretroviral treatment for HIV are infected with a strain that has been shown to be resistant to first-line HIV drugs such as efavirenz (WHO, 2020). Furthermore, infants born to mothers infected with HIV have been shown to have levels of HIV drug resistance as high as 69% (WHO, 2020).

HIV-1 Lentiviral Vectors

Viral vectors have been valuable assets to both gene therapy and viral research due to their ability to target specific cell types as well as their ability to integrate desired genes into the genomes of target cells through transduction. Early viral vectors were based on simple retroviruses that could only deliver genes into dividing cells, but recent use of viral vectors have shifted towards more complex viral vectors derived from the lentivirus genus of retroviruses, such as HIV-1, which can transduce both dividing and non-dividing cells (Liu and Berkhout, 2014). Furthermore, genes transduced by lentiviral vectors allow for stable gene expression in mammalian cell lines (Dautzenberg et al., 2021). Out of all currently available lentiviral vectors, HIV-1 derived lentiviral vectors have been the best characterized (Ailles and Naldini, 2002). Lentiviral vectors require the active transport of the preinitiation complex through the nucleopore using the nuclear import proteins of the host cell, which allows them to infect non-dividing cells (Kay et al., 2001). HIV-1 based lentiviral vectors often include a transfer vector with the transgene and separate packaging constructs (Liu and Berkhout, 2014). Both the transfer vector and the packaging constructs have underwent numerous revisions to increase biosafety of the vector, such as reducing the possibility of the vector becoming a replication-competent virus, and to increase transduction efficiency or transgene expression in host cells (Liu and Berkhout,

2014). Transfer vectors often include methods to distinguish transduced cells, such as a GFP expression cassette (Liu and Berkhout, 2014). Most lentiviral vector applications are also pseudotyped with the vesicular stomatitis virus-g (VSV-g) envelope protein, which is often provided through a separate vector (Liu and Berkhout, 2014). The VSV-g envelope provides vectors with expanded cellular trophism, increasing their applications in gene therapy and viral research (Kay et al., 2001; Liu and Berkhout, 2014).

Study Overview

There is a need to develop novel antiviral agents which can more effectively combat emerging resistant strains of HIV-1. This study will test 35 organic compounds and mixtures of organic compounds (collectively labeled BK compounds) on both their cytotoxicity as well as their ability to reduce HIV-1 expression in a mammalian cell line (CEMx174) that has been infected with HIV-1-D3-GFP vector. Compounds that have the potential to be used in HIV-1 treatments should exhibit a low cytotoxicity (cell viability greater than 90%) as well as high antiviral activity as visualized by the reduction of GFP expression. Compounds that exhibit these characteristics will be further screened to determine their EC_{50} (concentration that would inhibit the virus by 50%) and CC_{50} (cytotoxicity value that causes 50% cell population death). Ideally, compounds suitable for treatment should have low EC_{50} and high CC_{50}.

Materials and Methods

Cells

CEMx174 and HEK 293FT cells were cultured in RPMI 1640 medium (Corning) and DMEM (Gibco) respectively, which were supplemented with 10% FBS (Omega Scientific) and 1%

penicillin/streptomycin (Gibco). The cells were cultured at 37°C, 5% CO_2 according to the supplier's recommended subculturing method.

BK Compounds

All 35 BK compounds tested (BK 71-78, BK 80-BK 107) were provided by Dr. Jong Hyun Cho in Dong-a University's Laboratory of Antiviral Drug Discovery in Busan, South Korea. Compounds BK 71 to BK 78 were synthesized, BK 80 is a naturally occurring compound derived from the larvae of *Tenebrio molitor*, while BK 81 to BK 107 were mixtures of naturally occurring compounds extracted from various plants (see Appendix). The extraction of BK 80 was done using previously described techniques with the permission of the patent holders (Jun et al., 2018). Table 1 contains a summary of all compounds tested. Compounds with defined structures (BK 71-78, BK 80) were screened in concentrations of μM, while mixtures were screened in concentrations of μg/mL.

pD3HIV-1-GFP Vector Preparation

E. coli strains containing pVSV-g and pD3HIV-GFP genes, respectively, were cultured in 37°C for 12-16 hours, and the generated viral plasmids were isolated using isolated using the QIAGEN Plasmid Maxi Kit (Quiagen). The pD3HIV-1-GFP vector was prepared using methods previously described (Hollenbaugh et al., 2014). The vector encodes for a modified HIV-1 NL4-3 genome that had the HIV-1 *Nef* gene replaced with the eGFP (enhanced green fluorescent protein) gene and its *Env* gene deleted (Diamond et al., 2004). HEK 293FT cells were transfected with pD3HIV-GFP and pVSV-g using polyethyleneimine. On the third day, the media was collected for ultracentrifugation. The resulting pellet of virus-like particles (VLPs) was dissolved

in 700 μL of Hank's Balanced Salt Solution (Gibco) before being aliquoted and stored at -80°C until needed.

Cytotoxicity Assay

CEMx174 cells (150,000 cells/100 μL per well) were seeded in a 96-well plate in triplicates for each compound and mixture. After 24 hours, 100 μL of dissolved compound or mixture (20 μM or 20 μg/mL) was added in triplicates to their respective wells (final concentration of 10 μM or 10 μg/mL), with the negative control wells having no compound added. After 72 hours, the cells were collected according to the compound tested, and the viability of the cells were assessed using an MTS assay. The control groups, also in triplicates, included cells treated with 10 μM cycloheximide, a protein synthesis inhibitor with known cytotoxicity in mammalian cells (Schneider-Poetsch et al., 2010), and cells that received no treatment (cell control). The observed cell viability was normalized against the cell control. To determine compound cytotoxicity, we will plot the percent cell viability relative to cell control for all compounds and mixtures as well as the cycloheximide control (Figure 1). Cells over 90% cell viability relative to the cell control were deemed healthy.

Anti-HIV-1-D3-GFP Screening

CEMx174 cells (150,000 cells/100 μL) were seeded in triplicates for each compound and mixture in a 96-well plate. Then, 100 μL of each compound or mixture (20 μM or 20 μg/mL) was added into their respective wells (final concentration of 10 μM or 10 μg/mL). After 2 hours, 1 μL of pD3GFP vector was added to each of the applicable wells. Each plate tested included triplicate wells for a virus control, which had only pD3GFP vector added in its wells, as well as a

cell control, which had cells that received no treatment (cell control). Each plate also had triplicate wells for cells treated with pD3GFP vector and efavirenz, a non-nucleoside reverse transcriptase inhibitor effective in inhibiting HIV-1 (Wang et al., 2019). The plate was then left to incubate for 48 hours in 37°C, 5% CO_2 before each triplicate was collected into separate Eppendorf tubes and spun down at 3,000 x g for 5 minutes, washed with 3x 1 mL phosphate-buffered saline solution (Gibco) (3,000 x g for 5 minutes per spin). The resulting pellets were fixed in 150 μL each of 4% paraformaldehyde and kept in 4°C for 15 minutes before the formaldehyde was removed (spin at 3,000 x g for 5 minutes), and the resulting pellets were washed with another 1 mL PBS each. The resulting pellets were resuspended in 600 μL PBS + 12 μL FBS and stored in 4°C for fluorescence-activated cell sorting (FACS) analysis. A significant reduction in percent GFP expression relative to virus control corresponds to significant antiviral activity. GFP inhibition was normalized against the cell control. To determine the inhibition of the HIV-1-D3-GFP vector, we will plot the percent GFP expression relative to the virus control for each compound and the efavirenz control (Figure 2-4).

Determination of CC_{50} for BK 91

To determine the CC_{50} of BK 91, different concentrations of the mixture were tested in a cytotoxicity assay. We tested concentrations of 200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.25 μg/mL, 3.125 μg/mL, and 1.5625 μg/mL. The general procedure was the same as the one used for the cytotoxicity assay for BK 71 to BK 107, with the different concentrations of BK 91 taking the place of the different compounds/mixtures. The control groups included cells treated with 10 μM cycloheximide and a cell control group that received no treatment. To

determine the CC_{50}, we will plot the percent cell viability relative to cell control vs concentration of BK 91, and determine the concentration at which cell viability is 50% (Figure 5).

Determination of EC_{50} for BK 91

To determine the EC_{50} of BK 91, different concentrations of the mixture were tested in an anti-HIV-D3-GFP assay. Tested concentrations included 20 μg/mL, 10 μg/mL, 8.5 μg/mL, 6.5 μg/mL, 5 μg/mL, 2.5 μg/mL, and 1.25 μg/mL. The control group was a virus control, where only pD3GFP vector is added, and a cell control, which receives no treatment of compound or vector. The general procedure will be the same as the one used for the anti-HIV-D3-GFP assay for BK 71 to BK 107, with the different concentrations of BK 91 taking the place of the different compounds/mixtures. To determine the EC_{50}, we will plot the percent GFP expression relative to the virus control vs concentration of BK 91, and determine the concentration at which GFP expression is 50% (Figure 6).

Results

Results from the cytotoxicity assay (Figure 1) show that for all compounds and mixtures, the cell viability relative to the cell control after the addition of compound or mixture ranged from 93.6% to 102.8%. Since the cell viability relative to cell control was over 90% for all compounds and mixture, all compounds and mixtures tested did not have noticeable cytotoxicity. In the anti-HIV-1-D3-GFP screening, most of the compounds and mixtures did not show significant reduction in GFP expression (Figure 2 to Figure 4), as they had percent GFP expression similar to that of the virus control. However, BK 91, showed significant reduction in percent GFP expression (55.54 ± 1.10, $p<0.05$) relative to the virus control, but percent GFP expression that

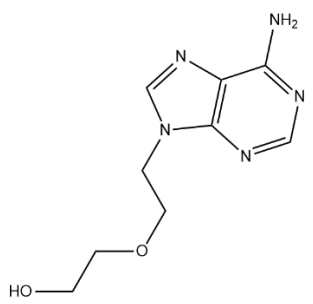
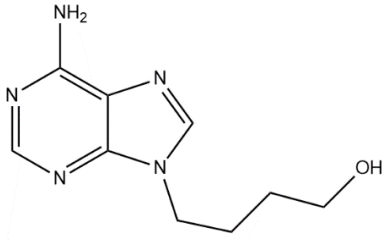
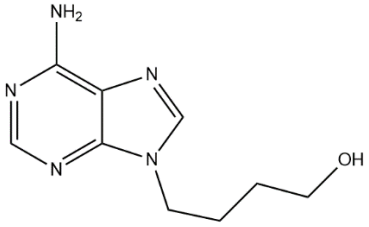
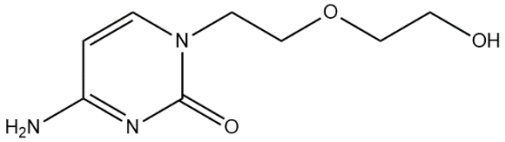
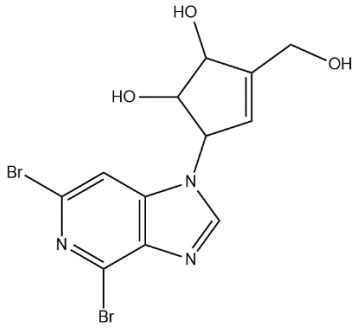
was significantly greater than that of efavirenz (24.25±1.71, $p<0.05$). Further in-depth analysis of BK 91 showed that the mixture has a CC_{50} value of approximately 57.47 μg/mL (Figure 5) and an EC_{50} value between 6.5 μg/mL and 8.5 μg/mL (Figure 6).

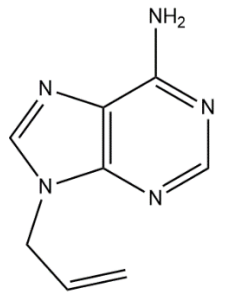
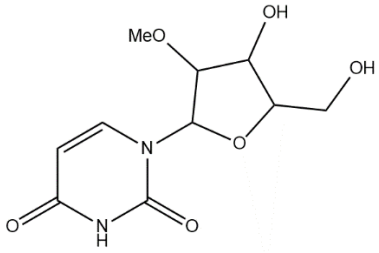
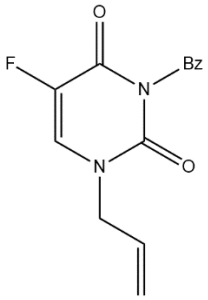
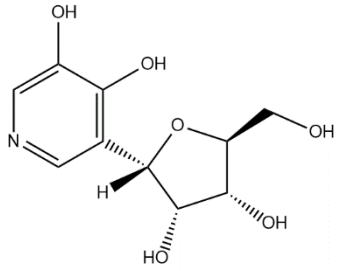
Discussion

Out of all compounds and mixtures tested in this study, mixture BK 91 exhibits promising characteristics that indicate it could be a suitable candidate for HIV-1 treatment out of all compounds and mixtures tested in this study. While all BK compounds tested indicated low cytotoxicity at concentrations of 10 μM (BK 71-78, BK 80) or 10 μg/mL (BK 81-107) with a cell viability over 90%, BK 91 is the only compound that showed significant reduction of percent GFP expression (55.54±1.10, $p<0.05$) in cells relative to that of the virus control. However, there are several limitations with this finding due to BK 91 is a mixture of organic compounds.

A major limitation with BK 91 is that due to being a mixture of organic compounds, we cannot deduce possible mechanisms of HIV-1 inhibition until we can identify the individual compounds that comprise BK 91. Consequently, being unable to identify potential mechanisms of inhibition also prevents us from categorizing BK 91 into a specific category of HAART drugs currently in use. Furthermore, future study will require the characterization of BK 91's individual compounds for both cytotoxicity and antiviral activity to identify which compounds that comprise BK 91 to narrow down the compounds that are responsible for the inhibition of the HIV-1-D3-GFP vector. Compounds that are identified to have both low cytotoxicity and antiviral activity will have their CC_{50} and EC_{50} determined and compared to the CC_{50} and EC_{50} of HAART compounds currently in use to determine if they have lower cytotoxicity or greater antiviral activity.

Tables and Figures

Compound	Structure	Molecular Weight (g/mole)
BK 71		223.23
BK 72		151.16
BK 73		207.23
BK 74		199.21
BK 75		405.04

BK 76		175.19
BK 77		258.23
BK 78		274.25
BK 80		243.21
BK 81	N1-EtOH	N/A
BK 82	N1-EA	N/A
BK 83	N1-MC	N/A
BK 84	N2-EtOH	N/A
BK 85	N2-EA	N/A

BK 86	N2-MC	N/A
BK 87	N3-EtOH	N/A
BK 88	N3-EA	N/A
BK 89	N3-MC	N/A
BK 90	N4-EtOH	N/A
BK 91	N4-EA	N/A
BK 92	N4-MC	N/A
BK 93	N5-EtOH	N/A
BK 94	N5-EA	N/A
BK 95	N5-MC	N/A
BK 96	N6-EtOH	N/A
BK 97	N6-EA	N/A
BK 98	N6-MC	N/A
BK 99	N7-EtOH	N/A
BK 100	N7-EA	N/A
BK 101	N7-MC	N/A
BK 102	N8-EtOH	N/A
BK 103	N8-EA	N/A
BK 104	N8-MC	N/A
BK 105	N9-EtOH	N/A
BK 106	N9-EA	N/A
BK 107	N9-MC	N/A

Table 1. Summary of compounds tested. Compounds BK 71 to 78 were synthesized, BK 80 is a naturally occurring compound extracted from the *Tenebrio molitor* larvae, and BK 81-107 were

mixtures of compounds extracted from various plants. For BK 81-107, characters before the hyphen indicates the plant (see Appendix), and characters after the hyphen indicates the solvent used to extract the compounds (EtOH: ethanol, EA: ethyl acetate, MC: dichloromethane).

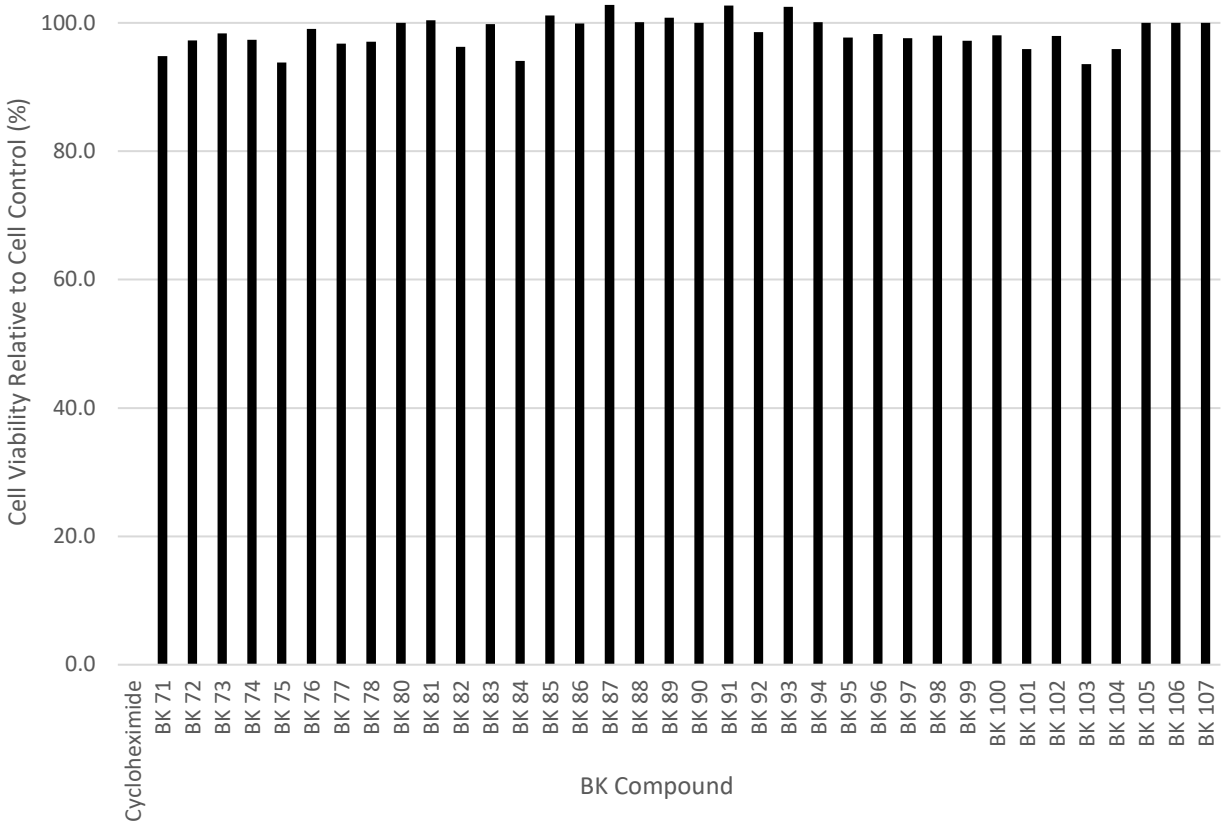


Figure 1. Results from cytotoxicity MTS assay. The graph shows the viability of CEMx174 cells treated with each compound. Cycloheximide is the positive control. Cell viability over 100% indicates that the cells in the treatment group had a higher viability than the cell viability of the control group.

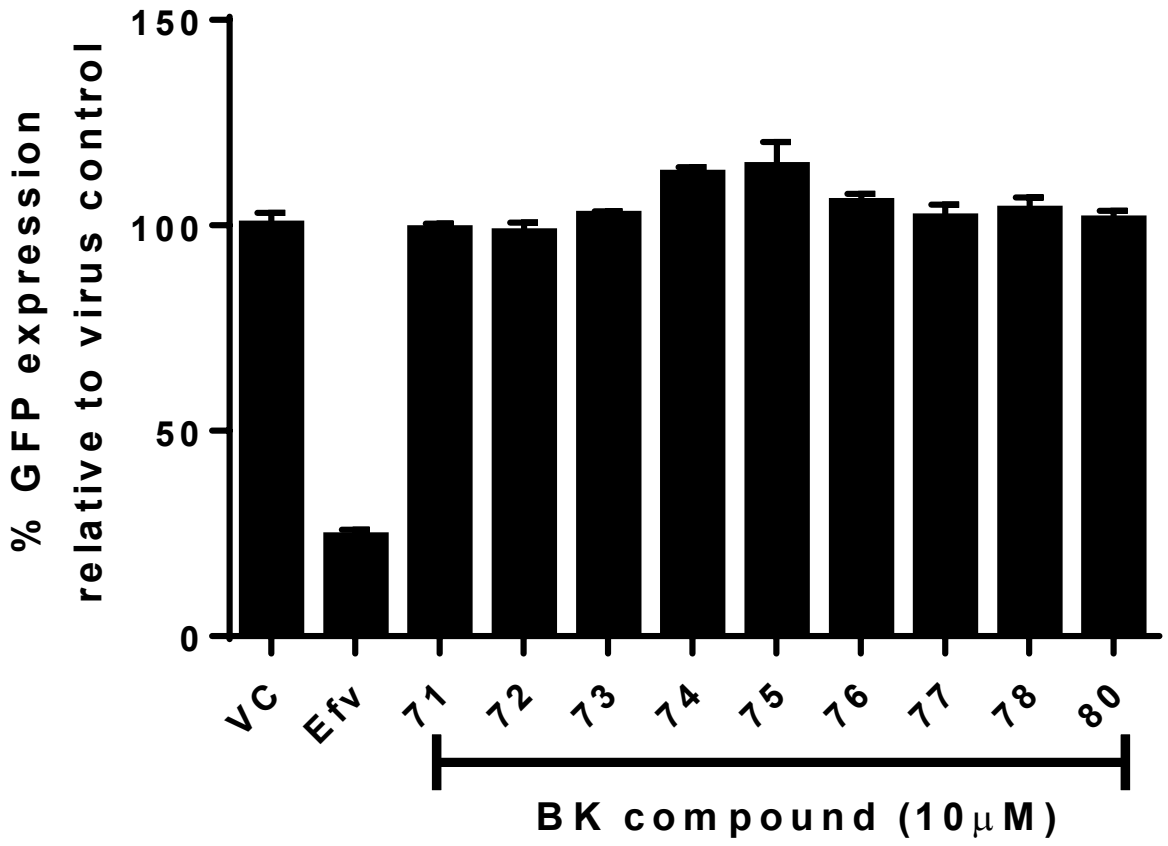


Figure 2. Results from HIV-1 antiviral assay (BK 71 to BK 80). VC indicates the GFP expression of the virus control, where only HIV-1-D3-GFP vector was added. Efv indicates the GFP expression of cells treated with both the viral vector and efavirenz.

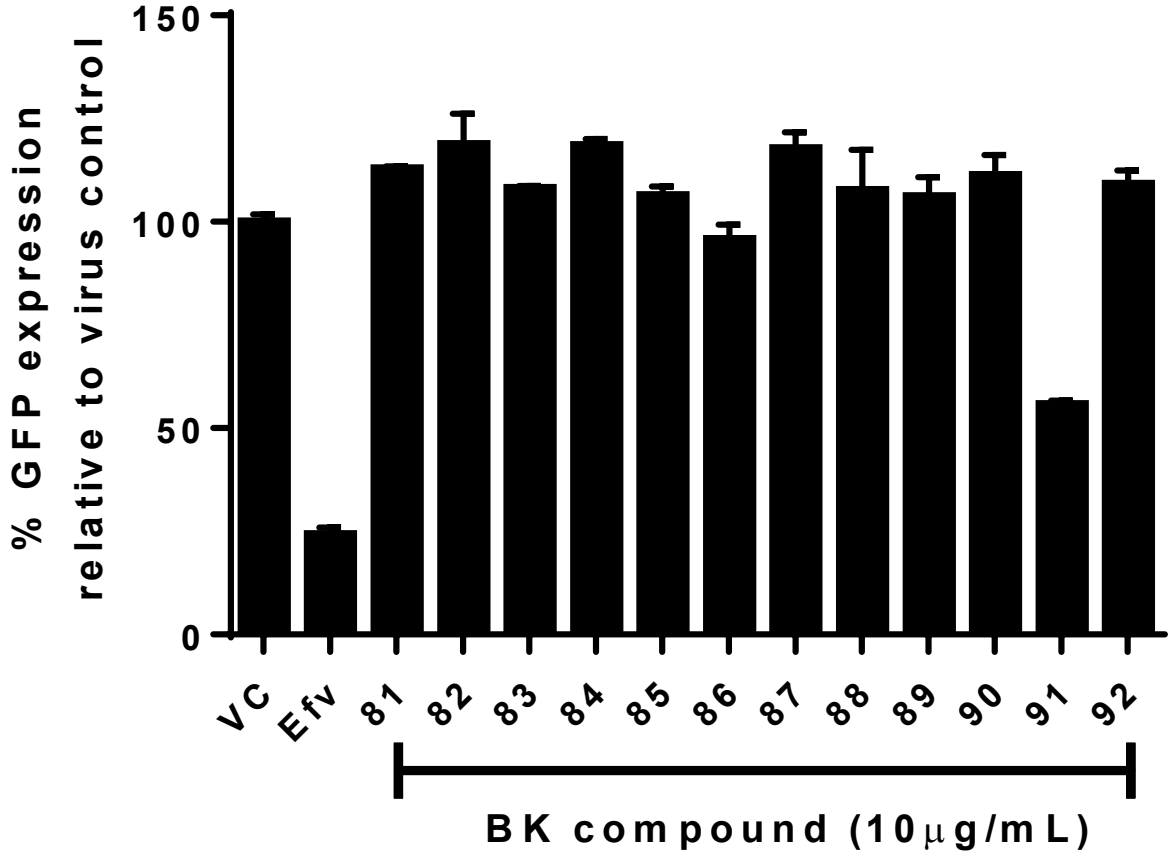


Figure 3. Results from HIV-1 antiviral assay (BK 81 to BK 92). VC indicates the GFP expression of the virus control, where only HIV-1-D3-GFP vector was added. Efv indicates the GFP expression of cells treated with both the viral vector and efavirenz. BK 91 displays significant reduction in percent GFP expression relative to viral control (55.65±1.10, $p<0.05$).

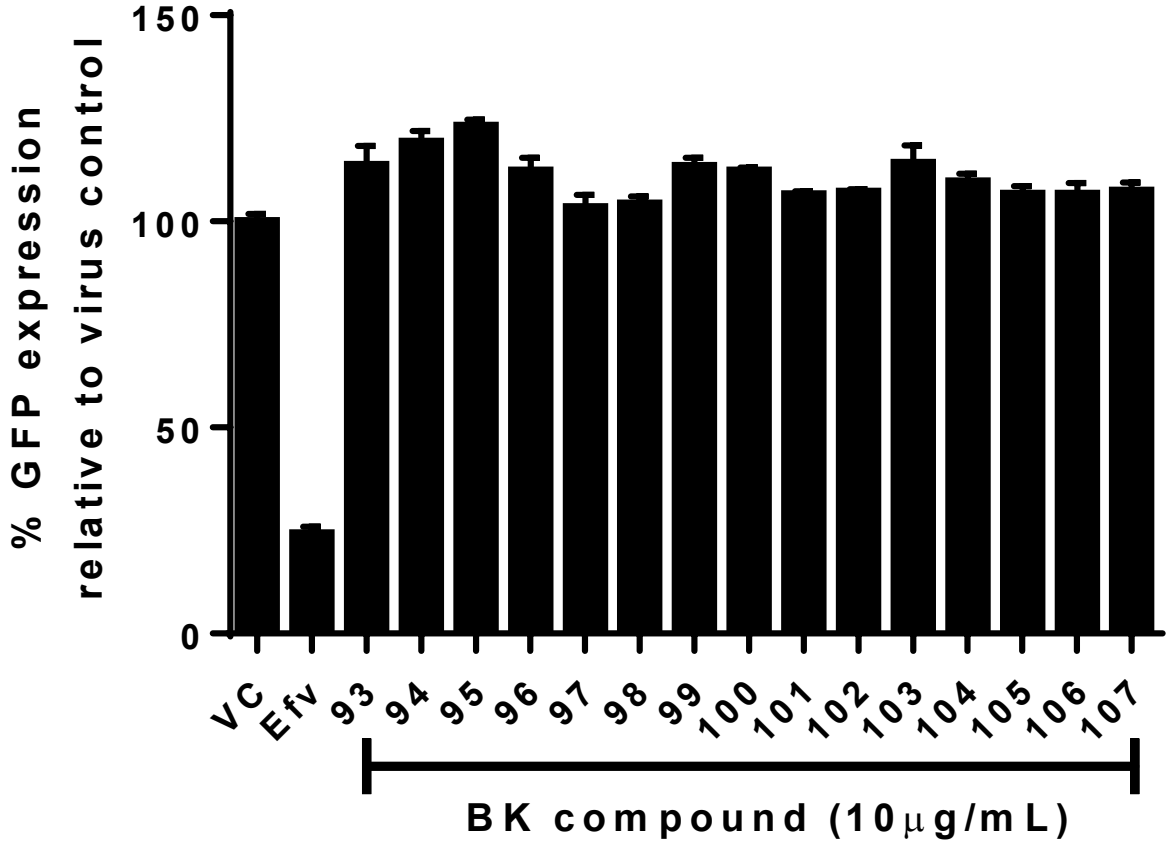


Figure 4. Results from HIV-1 antiviral assay (BK 93 to BK 107). VC indicates the GFP expression of the virus control, where only HIV-1-D3-GFP vector was added. Efv indicates the GFP expression of cells treated with both the viral vector and efavirenz.

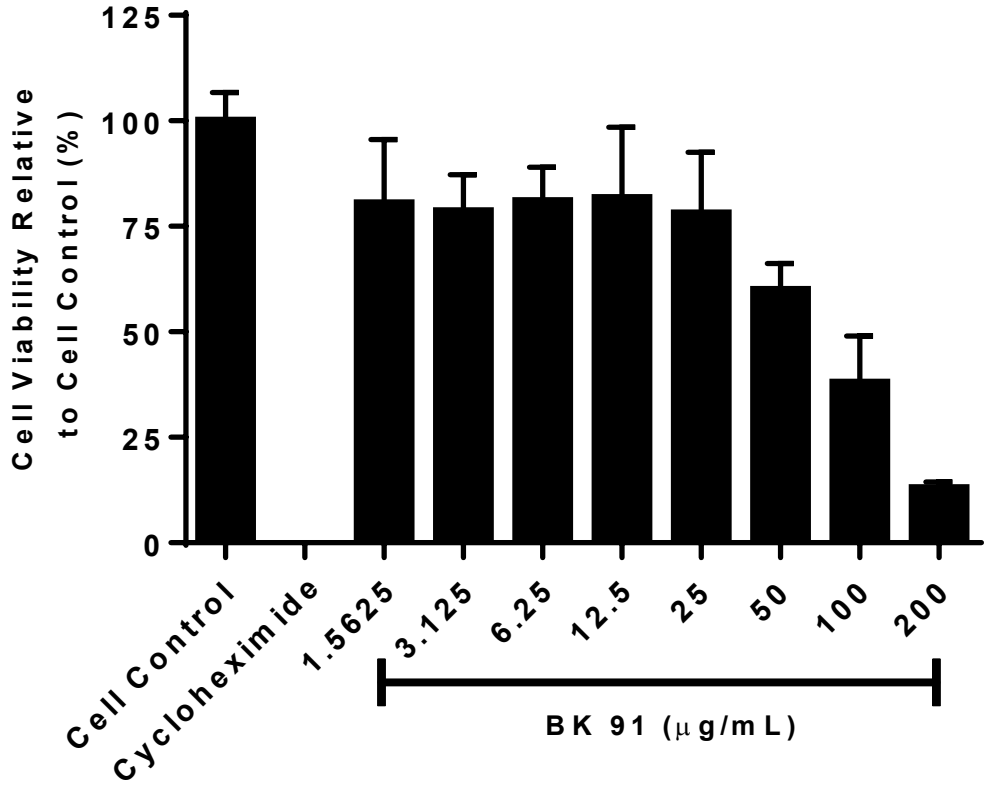


Figure 5. Results from determination of CC_{50} for BK 91. The CC_{50} value for BK 91 is between the concentration values of 50 μg/mL and 100 μg/mL, with an estimated value of (insert value here) μg/mL. The Cell Control group was not treated with any compound while the Cycloheximide group was treated with 10 μM of cycloheximide.

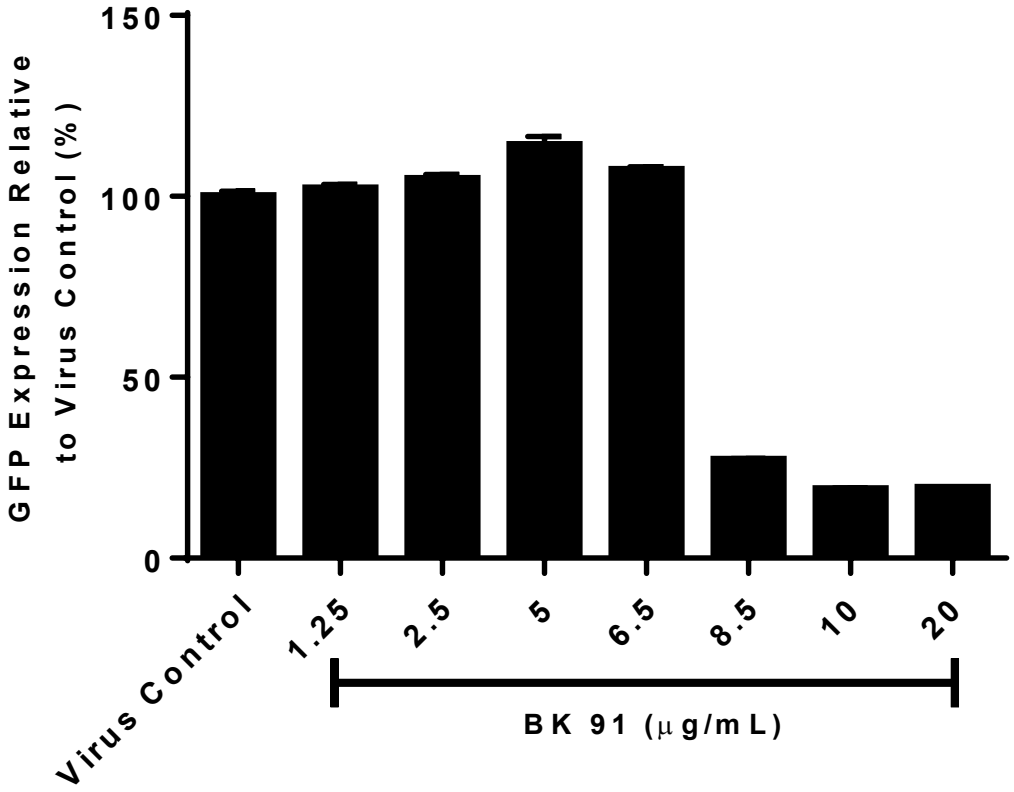


Figure 6. Results from determination of EC_{50} for BK 91. The EC_{50} for BK 91 is between the values of 6.5 μg/mL and 8.5 μg/mL, with an estimated value of (insert value here) μg/mL. The Virus Control group were only treated with the viral vector.

Appendix

Extraction of BK Compounds 81-107

The general procedure for the extraction of BK 81 to BK 107 is as follows: parts of plants were dried naturally before being crushed into powder weighing about 3 grams. Then, 100 mL solvent (ethanol (EtOH), dichloromethane (MC), or ethyl acetate (EA)) was added to the resulting powder. After stirring this mixture for 24 hours, the resulting solute was filtered and concentrated. Then, the concentrate was dried using a rotary evaporator to remove traces of solvent. The concentrate was dried further by a vacuum for 12 hours before collection. The origins of BK 81 to BK 107 include *Patrinia scabiosaefolia* (N1), *Thalictrum uchiyamai* (N2), *Thalictrum actaefolium* (N3), *Isodon japonicus* (N4), *Isatis tinctoria* (N5), *Rosa setigera* (N6), *Magnolia kobus* (N7), *Wisteria floribunda* (N8), and *Agastache rugosa* (N9). For preparation of N1, N2, N3, and N5, the entire plant without the root was used. For the preparation of N4 and N8, only the flower of the plant was used. For the preparation of N6 and N7, only the petals of the flower were used. For the preparation of N9, only the petals and the stem of the plant were used.

References

1. Ailles, L.E., and Naldini, L. (2002). HIV-1-derived lentiviral vectors. Curr Top Microbiol Immunol.
2. Aoki, M., Das, D., Hayashi, H., Aoki-Ogata, H., Takamatsu, Y., Ghosh, A.K., and Mitsuya, H. (2018). Mechanism of Darunavir (DRV)'s High Genetic Barrier to HIV-1 Resistance: A Key V32I Substitution in Protease Rarely Occurs, but Once It Occurs, It Predisposes HIV-1 To Develop DRV Resistance. mBio *9*, e02425-02417.
3. Battistini Garcia, S.A., and Guzman, N. (2020). Acquired immune deficiency syndrome CD4+ count (StatPearls Publishing LLC).
4. Beyrer, C., and Pozniak, A. (2017). HIV drug resistance — an emerging threat to epidemic control. New England Journal of Medicine *377*, 1605-1607.
5. Birkus, G., Hitchcock, M.J.M., and Cihlar, T. (2002). Assessment of mitochondrial toxicity in human cells treated with tenofovir: comparison with other nucleoside reverse transcriptase inhibitors. Antimicrobial Agents and Chemotherapy *46*, 716.
6. Bozzette, S.A., Ake, C.F., Tam, H.K., Chang, S.W., and Louis, T.A. (2003). Cardiovascular and Cerebrovascular Events in Patients Treated for Human Immunodeficiency Virus Infection. New England Journal of Medicine *348*, 702-710.
7. Brooks, J.T., Kaplan, J.E., Holmes, K.K., Benson, C., Pau, A., and Masur, H. (2009). HIV-associated opportunistic infections--going, going, but not gone: the continued need for prevention and treatment guidelines. Clin Infect Dis *48*, 609-611.
8. Das, S.R., and Jameel, S. (2005). Biology of the HIV Nef protein. Indian J Med Res.
9. Dautzenberg, I.J.C., Rabelink, M.J.W.E., and Hoeben, R.C. (2021). The stability of envelope-pseudotyped lentiviral vectors. Gene Therapy *28*, 89-104.
10. De Clercq, E. (2016). Tenofovir alafenamide (TAF) as the successor of tenofovir disoproxil fumarate (TDF). Biochemical Pharmacology *119*, 1-7.

11. De Meyer, S., Lathouwers, E., Dierynck, I., De Paepe, E., Van Baelen, B., Vangeneugden, T., Spinosa-Guzman, S., Lefebvre, E., Picchio, G., and de Béthune, M.-P. (2009). Characterization of virologic failure patients on darunavir/ritonavir in treatment-experienced patients. AIDS *23*, 1829-1840.
12. de Silva, T.I., Cotten, M., and Rowland-Jones, S.L. (2008). HIV-2: the forgotten AIDS virus. Trends Microbiol *16*, 588-595.
13. Debaisieux, S., Rayne, F., Yezid, H., and Beaumelle, B. (2012). The ins and outs of HIV-1 Tat. Traffic *13*, 355-363.
14. Deeks, E.D., and Keating, G.M. (2008). Etravirine. Drugs *68*, 2357-2372.
15. Diamond, T.L., Roshal, M., Jamburuthugoda, V.K., Reynolds, H.M., Merriam, A.R., Lee, K.Y., Balakrishnan, M., Bambara, R.A., Planelles, V., Dewhurst, S.*, et al.* (2004). Macrophage tropism of HIV-1 depends on efficient cellular dNTP utilization by reverse transcriptase. The Journal of Biological Chemistry *279*, 51545-51553.
16. Fanales-Belasio, E., Raimondo, M., Suligoi, B., and Buttò, S. (2010). HIV virology and pathogenetic mechanisms of infection: a brief overview. Annali dell'Istituto superiore di sanità *46*, 5-14.
17. Figueiredo, A., Moore, K.L., Mak, J., Sluis-Cremer, N., de Bethune, M.-P., and Tachedjian, G. (2006). Potent nonnucleoside reverse transcriptase inhibitors target HIV-1 gag-pol. PLOS Pathogens *2*, e119.
18. Fischl, M.A., Richman, D.D., Grieco, M.H., Gottlieb, M.S., Volberding, P.A., Laskin, O.L., Leedom, J.M., Groopman, J.E., Mildvan, D., Schooley, R.T.*, et al.* (1987). The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. New England Journal of Medicine *317*, 185-191.
19. Gazzard, B.G. (1995). Opportunistic infections in HIV seropositive individuals. J R Coll Physicians Lond *29*, 335-339.

20. Gelderblom, H.R., Özel, M., and Pauli, G. (1989). Morphogenesis and morphology of HIV structure-function relations. Archives of Virology *106*, 1-13.
21. German Advisory Committee Blood, S.A.o.P.T.b.B. (2016). Human immunodeficiency virus (HIV). Transfus Med Hemother *43*, 203-222.
22. Gerson, T., Makarov, E., Senanayake, T.H., Gorantla, S., Poluektova, L.Y., and Vinogradov, S.V. (2014). Nano-NRTIs demonstrate low neurotoxicity and high antiviral activity against HIV infection in the brain. Nanomedicine *10*, 177-185.
23. Hahn, B.H., Shaw, G.M., De, K.M., Cock, and Sharp, P.M. (2000). AIDS as a zoonosis: scientific and public health implications. Science *287*, 607.
24. Hajimahdi, Z., and Zarghi, A. (2016). Progress in HIV-1 integrase inhibitors: a review of their chemical structure diversity. Iran J Pharm Res *15*, 595-628.
25. Hattori, S.I., Hayashi, H., Bulut, H., Rao, K.V., Nyalapatla, P.R., Hasegawa, K., Aoki, M., Ghosh, A.K., and Mitsuya, H. (2019). Halogen bond interactions of novel HIV-1 protease inhibitors (PI) (GRL-001-15 and GRL-003-15) with the flap of protease are critical for their potent activity against wild-type HIV-1 and multi-PI-resistant variants. Antimicrob Agents Chemother *63*.
26. Hayashi, H., Takamune, N., Nirasawa, T., Aoki, M., Morishita, Y., Das, D., Koh, Y., Ghosh, A.K., Misumi, S., and Mitsuya, H. (2014). Dimerization of HIV-1 protease occurs through two steps relating to the mechanism of protease dimerization inhibition by darunavir. Proc Natl Acad Sci U S A *111*, 12234-12239.
27. Hemelaar, J., Gouws, E., Ghys, P.D., and Osmanov, S. (2006). Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS *20*, W13-W23.
28. Hicks, C., and Gulick, R.M. (2009). Raltegravir: The First HIV Type 1 Integrase Inhibitor. Clinical Infectious Diseases *48*, 931-939.

29. Hollenbaugh, J.A., Tao, S., Lenzi, G.M., Ryu, S., Kim, D.-H., Diaz-Griffero, F., Schinazi, R.F., and Kim, B. (2014). dNTP pool modulation dynamics by SAMHD1 protein in monocyte-derived macrophages. Retrovirology *11*, 63.

30. Hull, M.W., and Montaner, J.S.G. (2011). Ritonavir-boosted protease inhibitors in HIV therapy. Annals of Medicine *43*, 375-388.

31. Jun, M.R., Youn, K.J., Lee, S.A., Lee, S.E., and Yun, E.Y. (2018). Phamaceutical composition for treating degenerative brain disease contaning a compound isolated from Tenebrio molitor larvae (South Korea), pp. 22.

32. Jurado, K.A., Wang, H., Slaughter, A., Feng, L., Kessl, J.J., Koh, Y., Wang, W., Ballandras-Colas, A., Patel, P.A., Fuchs, J.R.*, et al.* (2013). Allosteric integrase inhibitor potency is determined through the inhibition of HIV-1 particle maturation. Proc Natl Acad Sci U S A *110*, 8690-8695.

33. Kahn, J.O., and Walker, B.D. (1998). Acute human immunodeficiency virus type 1 infection. New England Journal of Medicine *339*, 33-39.

34. Kalayjian, R.C., Lau, B., Mechekano, R.N., Crane, H.M., Rodriguez, B., Salata, R.A., Krishnasami, Z., Willig, J.H., Martin, J.N., Moore, R.D.*, et al.* (2012). Risk factors for chronic kidney disease in a large cohort of HIV-1 infected individuals initiating antiretroviral therapy in routine care. AIDS (London, England) *26*, 1907-1915.

35. Kang, D., Ruiz, F.X., Feng, D., Pilch, A., Zhao, T., Wei, F., Wang, Z., Sun, Y., Fang, Z., De Clercq, E.*, et al.* (2020). Discovery and characterization of fluorine-substituted diarylpyrimidine derivatives as novel HIV-1 NNRTIs with highly improved resistance profiles and low activity for the hERG ion channel. J Med Chem *63*, 1298-1312.

36. Kay, M.A., Glorioso, J.C., and Naldini, L. (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nature Medicine *7*, 33-40.

37. Kempf, D.J., Marsh, K.C., Kumar, G., Rodrigues, A.D., Denissen, J.F., McDonald, E., Kukulka, M.J., Hsu, A., Granneman, G.R., Baroldi, P.A.*, et al.* (1997). Pharmacokinetic enhancement of

inhibitors of the human immunodeficiency virus protease by coadministration with ritonavir. Antimicrobial agents and chemotherapy *41*, 654-660.

38. Koh, Y., Amano, M., Towata, T., Danish, M., Leshchenko-Yashchuk, S., Das, D., Nakayama, M., Tojo, Y., Ghosh, A.K., and Mitsuya, H. (2010). Selection of Highly Darunavir-Resistant and Replication-Competent HIV-1 Variants by Using a Mixture of Clinical HIV-1 Isolates Resistant to Multiple Conventional Protease Inhibitors. Journal of Virology *84*, 11961.
39. Kohler, J.J., Hosseini, S.H., and Lewis, W. (2008). Mitochondrial DNA impairment in nucleoside reverse transcriptase inhibitor-associated cardiomyopathy. Chem Res Toxicol *21*, 990-996.
40. Lee, F.J., Amin, J., Bloch, M., Pett, S.L., Marriott, D., and Carr, A. (2013). Skeletal muscle toxicity associated with raltegravir-Based Combination Antiretroviral Therapy in HIV-Infected Adults. JAIDS Journal of Acquired Immune Deficiency Syndromes *62*.
41. Lehman, D.A., Wamalwa, D.C., McCoy, C.O., Matsen, F.A., Langat, A., Chohan, B.H., Benki-Nugent, S., Custers-Allen, R., Bushman, F.D., John-Stewart, G.C.*, et al.* (2012). Low-frequency nevirapine resistance at multiple sites may predict treatment failure in infants on nevirapine-based treatment. J Acquir Immune Defic Syndr *60*, 225-233.
42. Li, G., Meanwell, N.A., Krystal, M.R., Langley, D.R., Naidu, B.N., Sivaprakasam, P., Lewis, H., Kish, K., Khan, J.A., Ng, A.*, et al.* (2020). Discovery and optimization of novel pyrazolopyrimidines as potent and orally bioavailable allosteric HIV-1 integrase inhibitors. J Med Chem *63*, 2620-2637.
43. Liu, Y.P., and Berkhout, B. (2014). HIV-1-based lentiviral vectors. In Human Retroviruses: Methods and Protocols, E. Vicenzi, and G. Poli, eds. (Totowa, NJ: Humana Press), pp. 273-284.
44. Lv, Z., Chu, Y., and Wang, Y. (2015). HIV protease inhibitors: a review of molecular selectivity and toxicity. HIV AIDS (Auckl) *7*, 95-104.
45. Makarov, E., Gerson, T., Senanayake, T., Poluektova, L., and Vinogradov, S. (2010). Efficient suppression of human immunodeficiency virus in macrophages by nano-NRTIs. Antiviral Research - ANTIVIR RES *86*.

46. Orkin, C., DeJesus, E., Khanlou, H., Stoehr, A., Supparatpinyo, K., Lathouwers, E., Lefebvre, E., Opsomer, M., Van de Casteele, T., and Tomaka, F. (2013). Final 192-week efficacy and safety of once-daily darunavir/ritonavir compared with lopinavir/ritonavir in HIV-1-infected treatment-naïve patients in the ARTEMIS trial. HIV Medicine *14*, 49-59.

47. Pau, A.K., and George, J.M. (2014). Antiretroviral therapy: current drugs. Infect Dis Clin North Am *28*, 371-402.

48. Poeschla, E.M. (2008). Integrase, LEDGF/p75 and HIV replication. Cellular and Molecular Life Sciences *65*, 1403-1424.

49. Sarafianos, S.G., Marchand, B., Das, K., Himmel, D.M., Parniak, M.A., Hughes, S.H., and Arnold, E. (2009). Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition. J Mol Biol *385*, 693-713.

50. Schneider-Poetsch, T., Ju, J., Eyler, D.E., Dang, Y., Bhat, S., Merrick, W.C., Green, R., Shen, B., and Liu, J.O. (2010). Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. Nature Chemical Biology *6*, 209-217.

51. Shafer, R.W., Kantor, R., and Gonzales, M.J. (2000). The genetic basis of HIV-1 resistance to reverse transcriptase and protease inhibitors. AIDS Rev *2*, 211-228.

52. Sohl, C.D., Singh, K., Kasiviswanathan, R., Copeland, W.C., Mitsuya, H., Sarafianos, S.G., and Anderson, K.S. (2012). Mechanism of interaction of human mitochondrial DNA polymerase γ with the novel nucleoside reverse transcriptase inhibitor 4'-ethynyl-2-fluoro-2'-deoxyadenosine indicates a low potential for host toxicity. Antimicrobial agents and chemotherapy *56*, 1630-1634.

53. Spagnuolo, V., Castagna, A., and Lazzarin, A. (2018). Darunavir for the treatment of HIV infection. Expert Opinion on Pharmacotherapy *19*, 1149-1163.

54. Taylor, B.S., Sobieszczyk, M.E., McCutchan, F.E., and Hammer, S.M. (2008). The challenge of HIV-1 subtype diversity. N Engl J Med *358*, 1590-1602.

55. Todd, J., Glynn, J.R., Marston, M., Lutalo, T., Biraro, S., Mwita, W., Suriyanon, V., Rangsin, R., Nelson, K.E., Sonnenberg, P.*, et al.* (2007). Time from HIV seroconversion to death: a

collaborative analysis of eight studies in six low and middle-income countries before highly active antiretroviral therapy. AIDS *21*.

56. UNAIDS (2020). Global HIV & AIDS statistics — 2020 fact sheet.
57. Van Heuverswyn, F., Li, Y., Neel, C., Bailes, E., Keele, B.F., Liu, W., Loul, S., Butel, C., Liegeois, F., Bienvenue, Y.*, et al.* (2006). SIV infection in wild gorillas. Nature *444*, 164-164.
58. Wang, Y., De Clercq, E., and Li, G. (2019). Current and emerging non-nucleoside reverse transcriptase inhibitors (NNRTIs) for HIV-1 treatment. Expert Opinion on Drug Metabolism & Toxicology *15*, 813-829.
59. WHO (2020). HIV drug resistance.