Screening for novel compounds produced by Actinobacteria and *Gardnerella vaginalis* that impact HIV-1 infection

By

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Abstract

HIV-1 infection continues to impact the lives of millions, with the most vulnerable being women living in Sub-Saharan Africa. Variation in the outcome of antiretroviral (ARV) and pre-exposure prophylaxis (PrEP) clinical trials is most likely multifactorial which could include Bacterial Vaginosis (BV), a condition associated with increased HIV acquisition and decreased tenofovir (TVF) efficacy. It is therefore important to not only understand how BV impacts HIV infection but also to identify alternative, novel ARVs. Natural products (NPs) have been harnessed and used as antibiotics, antifungals, and anti-virals and may represent a source of novel HIV drugs. This study determined whether NPs produced by selected filamentous actinobacteria and the BV-associated bacterium (BVAB) *Gardnerella vaginalis* (GV) could impact HIV infection. NPs from 23 actinobacteria strains were extracted and tested on pseudovirus (PSV) infection. Ten strains showed a range of anti-viral activity from 50 µg/mL to 25 µg/mL, and the identity of the NPs was determined through mass spectrometry and the Global Natural Products Social Molecular Networking (GNPS) database. These strains produced known antimicrobials: Actinomycin D, Valinomycin, Antimycin, Puromycin and Anisomycin. As some commercial antibiotics have been shown to have antiviral activity, we tested the ability of the antimicrobials to inhibit PSV infection. However, we were unable to determine accurate IC_{50} values due to the cytotoxicity of all the compounds. Further analysis is required to identify antimicrobial concentrations that are not cytotoxic but are still able to inhibit PSV infection.

Surprisingly, preliminary data indicated that GV, the most common bacterial species associated with BV, inhibited HIV infection (Unterpertinger and Abrahams, unpublished data). When PSV infection was measured in the presence of GV supernatant, PSV infection was significantly inhibited, suggesting that either the bacteria released a compound that impacted HIV infection or the culture medium, BHI was reducing infection. Ammonium sulphate (AS) precipitation was performed to isolate compounds from the supernatant of GV but, none of the fractions showed significant anti-viral activity. Subsequently, to confirm whether GV produced organic compounds that may be inhibitory, NPs were extracted from the abiotic culture medium using liquid-liquid extraction (LLE) and solid-phase extraction (SPE). A mid-polar fraction was identified that displayed anti-viral properties without reducing cell viability. After the fraction was analyzed by

mass spectrometry and screening of the GNPS database, Vitamin B_6 was identified as the possible inhibitor. Therefore, this study has identified compounds produced by filamentous actinobacteria and GV that might be novel inhibitors of HIV infection and further study is required to confirm their efficacy.

List of abbreviations

Full Name	**Abbreviation**
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
50 % tissue culture invective dose	$TCID_{50}$
5-fluoro-1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidine carboxylic acid	FOA
Approximately	~
Base pairs	bp
Calcium	Ca
Carbon dioxide	CO_2
C-C chemokine receptor type 5 CD4/8 Cluster of differentiation 4/8	CCR5
Cluster of differentiation 4	CD4
C-X-C chemokine receptor type 4	CXCR4
Degrees Celsius	°C
Deoxyribonucleic acid	DNA
Dimethyl sulfoxide	DMSO
Distilled water	dH_2O
Dulbecco's modified eagle medium	DMEM
Median Effective Dose	ED_{50}
Enzyme-linked immunosorbent assay	ELISA
Escherischia coli	*E. coli*
Ethyl Acetate	EtAc

Female genital tract	FGT
Fetal bovine serum	FBS
Global Natural Products Social Molecular Networking	GNPS
Hexane	C_6H_{14}
Human embryonic kidney 293T	HEK293T
Human immunodeficiency virus	HIV
HIV Drug Resistance	HIVDR
Dose of drug that results in 50% inhibition	IC_{50}
Cytotoxicity concentration at 50% of maximum	CC_{50}
Lethal Dose at 50% maximum	LD_{50}
Litres	L
Liquid-Liquid Extraction	LLE
Long-terminal repeat	LTR
Methanol	MeOH
Micrograms	µg
Microliters	µL
Micromolar	µM
Milliamps	mA
Millilitres	mL
Molar	M
Molecular weight	MW
Nanograms	ng

Nanometer	nm
Optical Density	OD
Phosphate buffered saline	PBS
Polymerase chain reaction	PCR
Pseudovirus	PSV
Relative centrifugal force	RCF
Relative light units	RLU
Revolutions per minute	rpm
Sodium chloride	NaCl
Sodium dodecyl sulfate	SDS
Solid Phase Extraction	SPE
Triple quadrupole time-of-flight instrument	QTOF
Tris(hydroxymethyl)aminomethane	Tris

Chapter 1: Literature Review

1.1 Introduction

Human Immunodeficiency virus (HIV) has infected 74.9 million people since the beginning of the pandemic, killing 32 million people globally(UNAIDS, 2019). HIV-1 is prevalent on almost every continent and is most commonly seen in men who have sex with men (MSM), transgender individuals, intravenous drug users and young female sex workers (UNAIDS, 2019). Sub-Saharan Africa carries the greatest burden of HIV-1 infection with 60% of all HIV-1 infections occurring in this region (Dwyer-Lindgren *et al.*, 2019) and an estimated 6 million people living with HIV/AIDS in South Africa alone. Millions are still newly infected every year and there is an increasing need for prevention strategies for those in high-risk regions (WHO, 2021). Current treatment for HIV-1 infection is known as HAART (Highly Active Antiretroviral Therapy). HAART commonly consists of a protease inhibitor and two nucleoside analogue reverse transcriptase inhibitors (Ghosn *et al.*, 2018).

Although antiretrovirals (ARVs) have made revolutionary steps in treating HIV infection and limiting the spread of the virus, the disease remains a global health challenge. This could be due to various factors, such as access to treatment, adherence, drug resistance, drug-drug interactions, and co-morbidities. Treatment with ARVs in the presence of other concomitant drugs has shown an impact on ARV efficacy due to drug absorption, metabolism and sometimes elimination of the drug completely, rendering ARVs less effective (OARAC, 2019). Poor adherence to HAART has resulted in resistance to ARVs (Hertogs *et al.*, 2000) and recent studies have reported increased resistance to Enfuvirtide, a fusion inhibitor (Chang *et al.*, 2021). There is thus a need to identify novel drugs that prevent HIV infection.

Over the last century, scientists have discovered, harnessed, and utilized NPs to solve and treat medical anomalies. NPs are chemical compounds isolated from diverse living things and are found in thousands of organisms (Bérdy, 2005). A famous example is the discovery of antibiotics in the 1940s and 1950s, such as Streptomycin, which later led to an exponential increase in NP discoveries worldwide (Waksman and Woodruff, 1940). With increased knowledge and technological advances, scientists have broadened the range of NPs found and used, allowing for the synthetic design of effective drugs and therapy for diseases. One of the most noteworthy phylum of NP producers are members of *Actinomycetota*, especially the filamentous members such as *Streptomyces* spp. due to the sheer number, versatility and diverse structures of metabolites produced. Known to produce hundreds of antibiotics and antifungals, filamentous actinobacteria also produce NPs that display anti-viral properties, begging the question of whether these microbes could be the solution to the current HIV-1 crisis (Chiba *et al.*, 2001; Liu *et al.*, 2013). By using anti-viral NPs, one may be able to quickly and effectively produce novel anti-HIV drugs that can bypass drug resistance, drug-drug interactions. They could also be effective for women with Bacterial Vaginosis (BV) who are the most vulnerable cohorts to HIV-1 infection.

Bacterial Vaginosis is characterized by the outgrowth of anaerobic bacteria such as *Gardnerella vaginalis* (GV) with concomitant loss of *Lactobacillus* spp. of the female genital tract (FGT) (Randis and Ratner, 2019). Metanalysis of 23 publications have shown that BV is associated with increased HIV acquisition (Atashili *et al.*, 2008) and the presence of dysbiotic bacteria in the FGT has been found to reduce ARV efficacy *in vitro* (Taneva *et al.*, 2018). Changes in the FGT microbiome could contribute to the variation observed across multiple pre-exposure prophylaxis (PrEP) studies conducted on female-only cohorts. For example, the FEM-PrEP study showed that once-daily Truvada had no impact on HIV infection in HIV-negative African women (Van Der Straten *et al.*, 2012) whereas the TDF2 study showed that once-daily Tenofovir Disoproxil Fumarate (TDF) prevented acquisition in women with 63% efficacy (Thigpen *et al.*, 2012).

The exact mechanism whereby BV impacts HIV acquisition remains unknown but as GV has been linked to BV (Morrill *et al.,* 2020) it has been suggested that these bacteria could also play a role in HIV infection (Cohen *et al.*, 2012). Not all BV-positive women have GV and because of its high genetic diversity, it has been suggested that strains vary in pathogenicity (Alves *et al.*, 2014). Therefore, it is important to understand the role of GV in the context of HIV-1 infection and identify potential mechanisms whereby GV, and potentially BV, enhances HIV acquisition.

Identification of compounds produced by GV that impact HIV infection could be targets for the design of novel drugs that might be able to prevent BV from increasing HIV acquisition.

1.2 HIV

As of 2021, 38.4 million [33.9 million–43.8 million] people globally were living with HIV and 650 000 people died from HIV-related illnesses in 2021 alone (WHO, 2021)HIV is grouped into two types: HIV type-1 (HIV-1) and HIV type-2 (HIV-2), and both are members of the *Retroviridae* family of the *Lentivirus* genus (Gigantesco and Guiliani2011). HIV-2 is found in remote regions of Western and Central Africa. HIV-1 is subdivided into 4 groups (M, O, N and P) where group M is further classified into 9 subtypes. HIV-1 group M viruses are responsible for the pandemic but due to its high diversity, the development of a successful vaccine is highly challenging. Even with effective ARV administration, the high mutation rate of the virus can lead to drug resistance and result in the drug no longer being effective.

1.3 HIV structure and replication

HIV contains two copies of single-stranded RNA, which consist of three main structural genes: *gag, pol* and *env* as well as several accessory genes *vif, vpr, vpu, tat, nef* and *rev* (Figure 1.1).

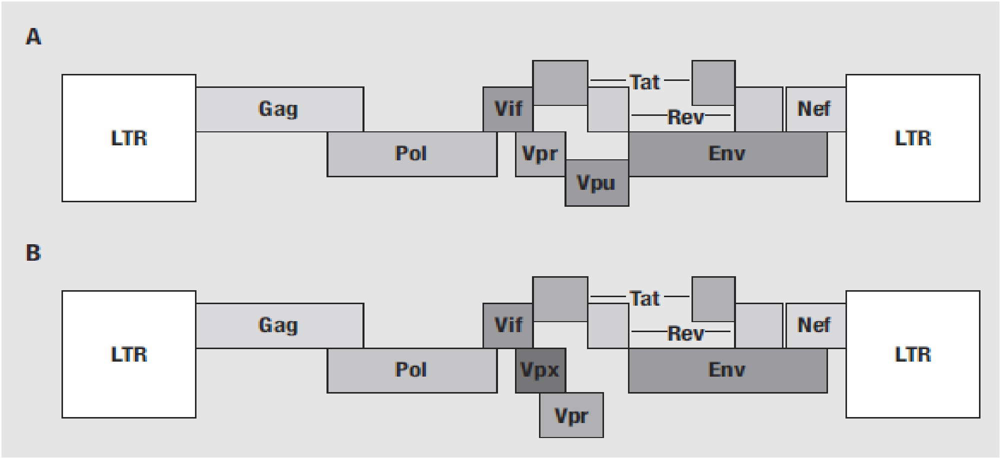

Figure 1.1: Diagram depicting the genome structure of HIV-1 (A) and HIV-2 (B) (Gigantesco and Guiliani2011).

As reviewed by Gigantesco and Guiliani(2011), the structure of HIV-1 such as that of other retroviruses; the *gag* gene encodes the core structural proteins (namely p24, p6 and p7) as well as the matrix (p17). The *pol* genes encode proteins essential for viral replication, such as Reverse Transcriptase (RT), Integrase (Int) and Protease (Prot). The *env* gene encodes the viral Envelope (Env) glycoproteins (gp120 and gp41). These proteins bind to cell surface receptors present on the surface of CD4+ T-lymphocytes, T-cell precursors, monocytes, dendritic cells and microglial cells of the central nervous system (Dean *et al*., 1996). Viruses that bind to the CCR5 co-receptor, commonly present on monocytes/macrophages, dendritic cells and activated T-lymphocytes, are known as R5-tropic HIV (Hill *et al*., 1997). Viruses that bind to the CXCR4 co-receptor, found on many cells including T-lymphocytes, are known as X4-tropic HIV (Feng *et al.*, 1996). Viruses which bind to both are named R5X4 HIV (Figlar,2006).

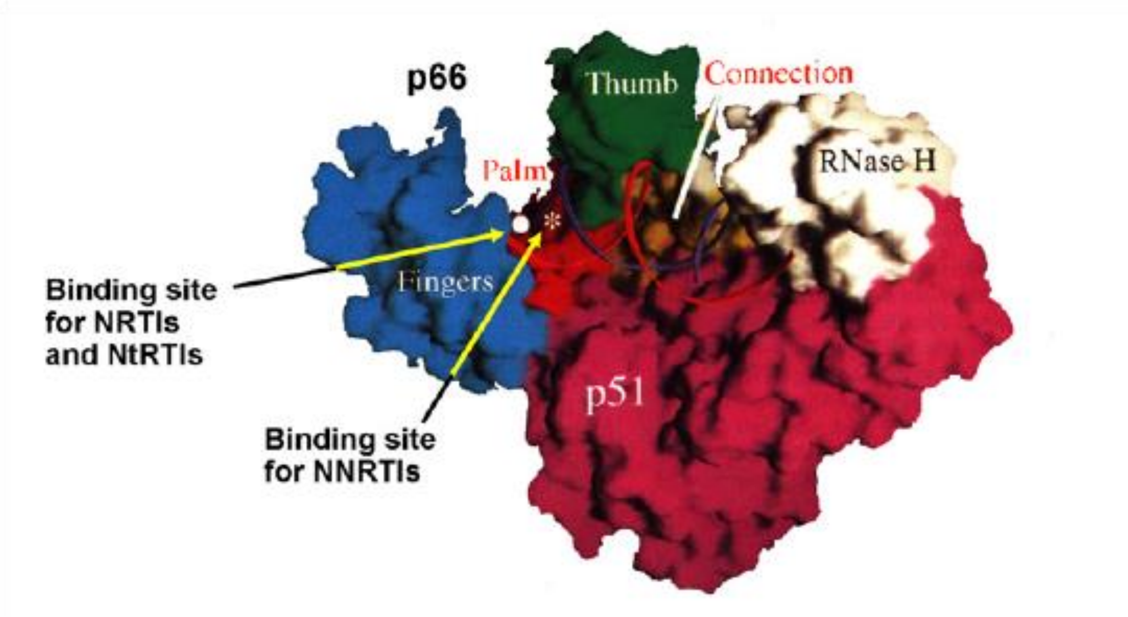

Figure 1.2: Diagram of Reverse Transcriptase (RT) and the target sites of NRTIs, NtRTIs and NNRTIs (De Clercq, 2009)

HIV replication occurs in six steps, namely: binding and entry, uncoating, reverse transcription, provirus integration, virus protein synthesis and assembly and finally budding (Figure 1.3) (Gigantesco and Guiliani 2011). During entry, gp120 binds to the CD4 receptor present on the host cell. When this binding occurs, the viral envelope undergoes a conformational change, allowing gp120 to expose the binding site for CCR5 or CXCR4 co-receptors, depending on tropism

(Wyatt R. *et al.*, 1998). Once this ‘double-binding’ has occurred, gp41 can now penetrate the cell membrane. During this penetration, gp41 undergoes a conformational change, forming a six-helix bundle that results in membrane fusion (Chan *et al.*, 1997). This allows the virus to enter the cytoplasm and uncoat so that the viral RNA can be reverse transcribed by RT. RT comprises of ribonuclease (RNaseH) and polymerase activity. It is composed of two subunits, p51 and p66, where the polymerase and RNase H reside on the p66 subunit. The p66 subunit contains the active sites for the enzymatic roles of RT. The p51 subunit plays a supportive role. The whole enzyme contains fingers, palm and a thumb (Figure 1.2). The overall structure of RT is elongated, with the polymerase active site near one end and the RNase H 60 Å away at the other end (Tian *et al.*, 2018).

Briefly, lysyl tRNA binds to a primer site on the minus strand of RNA and RT adds nucleotides using RNA as a template to form an RNA/DNA hybrid double helix. The RNaseH active site breaks down the RNA strand and the polymerase completes a complementary strand of DNA, resulting in a double-stranded DNA helix (Gigantesco and Guliani2011). This is then integrated into the host genome by Int and by essentially ‘hijacking’ the host’s transcription and translation machinery, viral proteins are expressed and then cleaved by Prot. Viral components are trafficked to the cell membrane, processed and particles are assembled before budding from the surface of the cell.

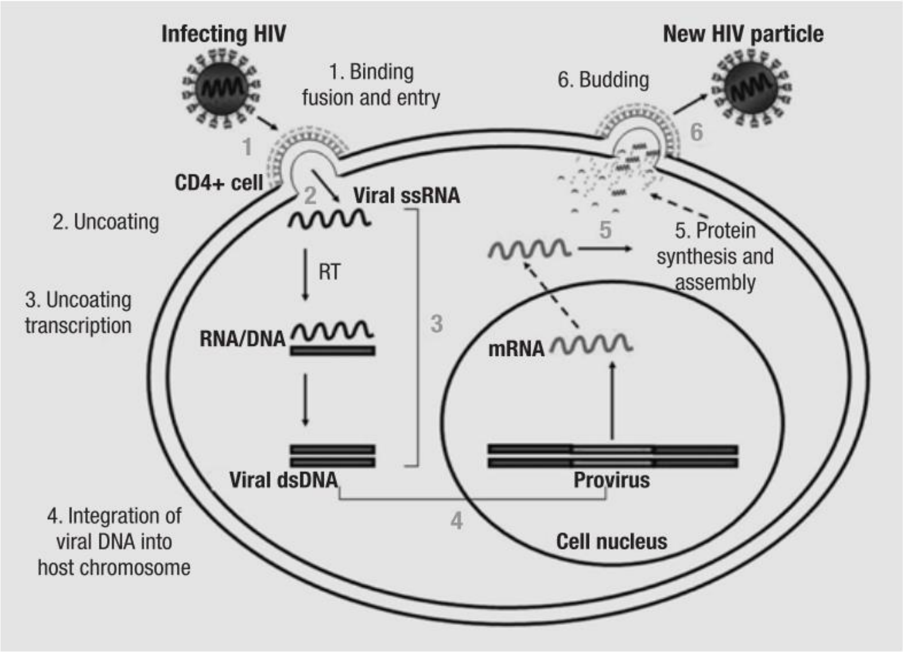

Figure 1.3: Diagram of HIV-1 replication in a CD4+ cell (Gigantesco and Guliani, 2011)

1.4 ARVs and their modes of action

Current treatment for HIV-1 infection, HAART, comprises of a regimen of ARVs prescribed to HIV-positive patients. HAART in South Africa includes a formula based on the fixed dose combination (FDC) of Tenofovir Disoproxil Fumarate (TDF) at 300 mg with Lamivudine (3TC) at 300 mg and Dolutegravir (DTG) at 50 mg (also known as TLD) for all eligible adults, adolescents and children over 10 years of age and weighing 35 kg and more (South African National Department of Health, 2019). However, recent studies have shown that DTG leads to neuropsychiatric side effects such as depression, insomnia and abnormal dreams (Kolakowska *et al.*, 2019; Murrell *et al.*, 2019). The use of DTG has also shown to lead to weight gain, and with the most vulnerable group being African women, could lead to psychological issues as well as hypertension (Calmy *et al.*, 2020). Additionally, DTG has shown to increase creatinine levels by inhibiting the organic cation transporter 2 (OCT2) on the basolateral side of proximal tubular cells (Wagner *et al.,* 2016) and if used with TDF, which is known to be nephrotoxic, could lead to renal damage (Fernandez-Fernandez *et al.*, 2011; Kolakowska *et al.*, 2019).

There are several types of ARV classes that target different aspects of viral replication and have different mechanisms of action. Tenofovir (TVF) is a nucleotide reverse transcriptase inhibitor (NtRTI). TVF resembles a nucleotide however once it is incorporated into the growing DNA strand it blocks elongation during transcription, terminating transcription (Figure 1.4) (De Clercq,2009). It is an acyclic analog of deoxyadenosine 5'-monophosphate (d-AMP) but lacks a hydroxyl group in the 3' position corresponding to the 3' carbon of d-AMP. This prevents the formation of the 5' to 3' phosphodiester link necessary for DNA elongation. TVF is phosphorylated to tenofovir diphosphate, the active form of the drug which terminates DNA transcription and prevents viral replication (Fung *et al*., 2002). TDF is a prodrug and is hydrolyzed enzymatically *in vivo* to TVF which is then phosphorylated to the active form, tenofovir di-phosphate. Therefore, TDF and TVF have the same mechanism of action.

NtRTIs interact with the active site of RT, while non-nucleoside reverse transcriptase inhibitors (NNRTIs) interact with an allosteric site a short distance away from the active site (Figure 1.2) (De Clercq,2009). However, NtRTIs have been associated with severe lactic acidosis and hepatomegaly with steatosis (Grim and Romanelli,2003). Patients prescribed with TDF have also noted rashes, diarrhea, depression and nausea (Wassner *et al.,* 2020). An additional disadvantage to NtRTIs is that they only act on the virus after infection which might be too late to clear the infection. Once the virus genome integrates within the human chromosome there is a possibility ffof a latent reservoir and thus chronic infection.

Therefore, ARVs such as Maraviroc (MV) might be more beneficial as they target viral entry and thus prevent virus infection. MV is a co-receptor inhibitor (CRI) and prevents viral entry by binding to CCR5 receptors on the surface of macrophages and T-cells (De Clercq, 2009). Similarly, drugs such as Aplaviroc, Vicriviroc, TAK-220 and PRO-140 also target CCR5 and bind to the transmembrane domains and these have not yet been linked to resistance mutations due to the native structure of the protein being essential for functionality (Poveda and Soriano 2006). Although effective, these drugs can only target CCR5 receptors, therefore X4-tropic HIV will not be inhibited. However, there are numerous CXCR4 antagonists being developed, such as AMD070, and KRH-1636/KRH-2731 that bind to the second and third loops of CXCR4, preventing entry, therefore effective against X4-tropic HIV (Poveda and Soriano, 2006). Another pivotal drug is Enfurvitide (T20), which is a fusion inhibitor (FI). This polypeptide is homologous to the heptad repeat (HR) regions of gp41 and when bound, forms a coiled-coil interaction, preventing fusion of the viral and cellular membranes (De Clercq, 2009).

HAART regimens vary from region to region, but even when similar regimens are employed different population groups display variation in resistance. High-income cohorts receiving TVF as a first-line regimen showed low TVF resistance mutations whereas in low and middle-income countries, patients receiving TVF as a first-line regimen showed high levels of resistance mutations, specifically a RT K65R mutation (Sunpath *et al.*, 2012). High levels of resistance against NNRTIs such as emtricitabine and lamivudine, which are components of the South African HAART regimen, are also prevalent in low to middle income countries, with the mutation only being a single amino acid change (Gregson *et al.*, 2016). This, as well as the mentioned adverse side effects, emphasizes the need for new drugs, especially for populations based in Sub-Saharan Africa.

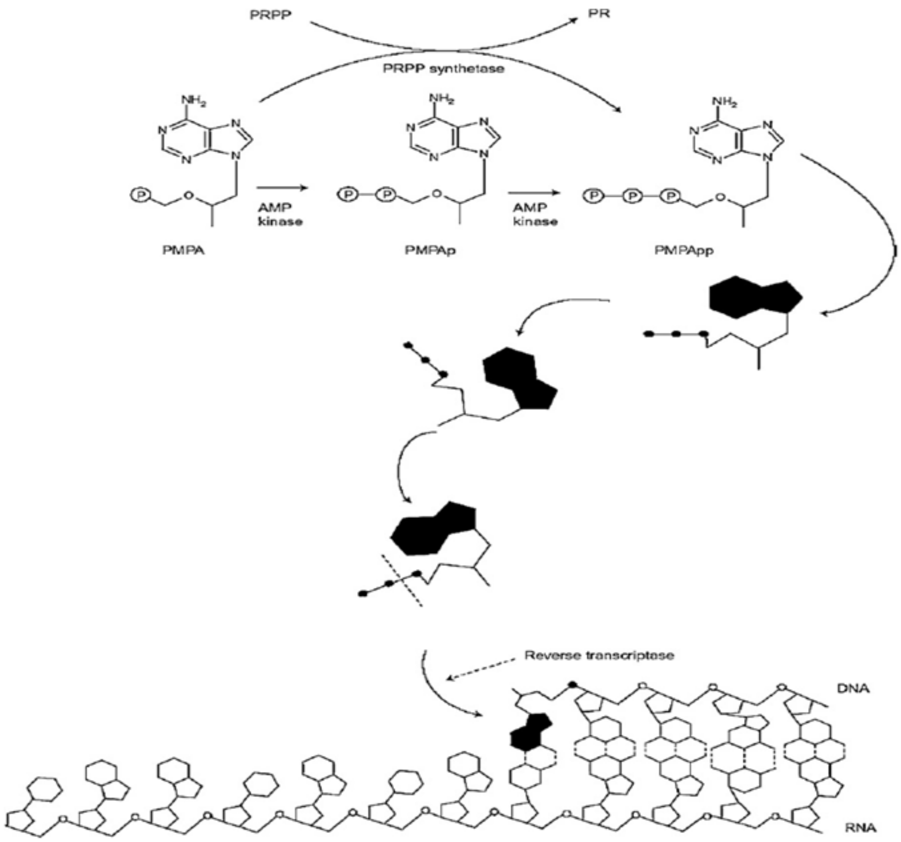

Figure 1.4: Diagram depicting mechanism of action of TVF during reverse transcription. TVF (or PMPA (R-)9-(2-phosphonylmethoxylpropyl) adenine) must be phosphorylated twice to be activated and then binds to the catalytic site of RT, terminating elongation (De Clerq,2009)

1.5 HIV-1 drug resistance and drug-drug interactions

The rapid mutation rate due to high replication with little to no error-repair has allowed HIV-1 to evolve and adapt to past and current treatment. Currently, more than 200 mutations are associated with ARV resistance to drugs belonging to six licensed ARV classes (Wensing *et al.*, 2017). Even with the introduction of new drugs, such as the CCR5 and Int inhibitors in 2007, mutations have increased resulting in greater drug resistance. In the context of CCR5, MV allosterically inhibits HIV-1 gp120 binding to the CCR5 receptor. However, it has been seen that HIV-1 displays two phenotypic mechanisms of resistance to the drug: enhanced binding to unbound CCR5 receptors (Pugach *et al.*, 2007) or enhanced binding to CCR5-inhibitor complexes regardless of drug concentration (Westby *et al.*, 2007). MV, which is used in the US in first- and second- line regimens, is prescribed as a salvage drug in South Africa after treatment failure with initial regimens (Meintjes *et al.*, 2014). However, as more CXCR4-tropic variants have been identified in chronically infected individuals in South Africa, MV might not be an optimum salvage therapy (Matume *et al.*, 2018). In low to middle-income countries, 9.7% of adults on ARVs have HIV drug resistance (HIVDR) predominantly against NNRTIs in Southern and Western Africa (Gupta *et al.*, 2018). In a recent study, 200 subjects (27.4%) showed HIVDR against NNRTIs (Moyo *et al.*, 2020), suggesting that current ARVs are no longer effective within the context of low to middle-income countries such as South Africa, and will only lead to increased morbidity and mortality.

However, resistance to current ARVs is not the only issue. Pharmacokinetic interactions and their impact on ARV efficacy are another reason better treatment is needed. HAART includes multiple ARVs to ensure increased efficacy (Atta *et al.,* 2019), however drug-drug interactions could directly mitigate the advantages. For example, in 2019, the HIV co-infection rate among notified TB cases in South Africa was 59% (Van der Walt *et al.*, 2018). Five drugs that constitute the recommended and prescribed regimen inhibit major metabolic pathways of ARVs, including Cytochrome P450 enzymes (CYP) CYP3A4, CYP2B6, CYP2C9 and CYP219 (Mukonzo *et al.*, 2019). CYP enzymes are vital for drug metabolism and the interaction between ARVs and their corresponding CYP enzymes plays an integral role in ARV efficacy. For example, Protease Inhibitors (PIs) inhibit CYP3A4 allowing for a slow release of other ARVs, reducing the frequency of drug intake by the patient (Yallapu *et al.*, 2020). Rifampicin, a prescribed TB drug, induces CYP enzymes and P-glycoproteins which decrease the concentration of NNRTIs and Pis, decreasing ARV efficacy and increasing viral resistance (McIlleron *et al.*, 2007). These pharmacokinetic contraindications emphasize the need for novel ARVs whose efficacy is not hindered by other drugs necessary to treat prevalent infectious diseases. Additionally, HIV-positive patients with kidney-related issues require a specific dosage of ARVs, which compromises drug efficacy (Swanepoel *et al.*, 2018). Furthermore, although the development of the new NNRTI Rilpivirine (RPV) in combination with ETC and TDF has shown promising results and efficacy, this regimen must be prescribed with caution if a HIV-positive patient has underlying heart issues.

1.6 PrEP variability and safety

Numerous studies have measured the efficacy of preexposure prophylaxis (PrEP) *in vitro, ex vivo*, in non-human primate models and in human trials (Table 1). Studies such as CAPRISA 004, FEM-PrEP, FACTS-001, VOICE and many others tested various regimens of TVF, TDF, TDF/Emtricitabine (TDF-FTC) or 1% TVF microbicide. Results from the iPrEx phase III trial showed that once-daily TDF-FTC reduced the risk of HIV-1 infection in homosexual men, transgender women and other MSM by 43.8% (Grant *et al.*, 2011).However, results from the FACTS-001 study showed that none of the ARV regimens reduced the rates of HIV-1 acquisition in African women (Delany-Moretlwe *et al.*, 2018). Heneine and Kashuba(2012) suggested that mucosal penetration by ARVs, targeting the right cells, dosing with the right concentrations and finding the right time to give the drugs to the patient contributed to the variation between studies (Heneine and Kashuba2012). Outcomes of studies could also be affected by characteristics of cohorts as some studies compared PrEP efficacy only amongst heterosexual women (Chirenje *et al.,* 2010) whereas others compared efficacy amongst MSM, transgender women and heterosexual women (Liu *et al.*, 2010). Furthermore, differences in methods of application could also play a role because PrEP can be taken orally, such as TDF or TDF-FTC, or it can be used as a microbicide gel (Engle *et al.*,2008). The MTN-001 Randomized Pharmacokinetic Cross-Over study by Hendrix *et al.* (2013) compared TDF pharmacokinetics in blood, vaginal tissue and rectal fluid in 144 HIV-uninfected women after either oral or vaginal dosing. Hendrix *et al.* (2013) showed that vaginal dosing of this PrEP resulted in a higher concentration of the drug in the FGT compared to levels found in serum. Therefore, vaginal administration provided a higher active site concentration, making it a potentially better form of PrEP for women compared to oral administration (Hendrix *et al.*, 2013). Interestingly, the FACTS 001 trial found the pericoital microbicide ineffective in protecting women against HIV infection. The study emphasized the need for a regimen that is “less user dependent” and that did not require high levels of adherence. However, a possible explanation for the ineffectiveness of the gel was the influence of cervicovaginal inflammation as well as the FGT microbiota (Delany-Moretlwe *et al.,* 2018). Both factors were known to influence TVF concentrations and could have affected the efficacy of the gel at the site of application.

Table 1.1: Examples of clinical trials investigating oral ARVs for HIV prevention

Authors	**Trial**	**Cohort**	**Outcome**
Robert M Grant (2010)	iPrEX	2499 HIV-seronegative men or transgender women who have sex with men	Once-daily Truvada reduced risk of HIV infection by an average of 44%
Lut Van Damme (2012)	FEM-PrEP	HIV-negative African women	Once-daily Truvada did not seem to decrease the risk of HIV infection among HIV-negative women
J.M. Baeten (2012)	Partners PrEP	HIV-1–serodiscordant heterosexual couples from Kenya and Uganda	Once-daily oral TDF and TDF-FTC was effective in preventing acquisition in both men and women
Thigpen *et al* (2012)	TDF2	HIV-seronegative men and women from Botswana	Once-daily oral TDF-FTC prevented acquisition in both men and women with 63% efficacy
Chirenje *et al*(2010)	MTN 003 (VOICE)	HIV-negative African women	Oral TVF and 1% TVF gel was tested. The 1% TVF gel was terminated due to a lack of effect relative to placebo

1.7 Bacterial Vaginosis

Bacterial Vaginosis is caused by an imbalance of 'healthy' vaginal lactobacilli and other complex, anaerobic bacteria such as GV and *Prevotella bivia* (Srinivasan *et al.*, 2010). The results of the CAPRISA 004 study showed that the efficacy of the 1% TVF microbicide was greater in African women with high levels of lactobacilli spp. in their FGT compared to women with a FGT microbiota dominated by non-lactobacilli spp.(Table 2) (Abdool Karim and Abdool Karim,2010). Subsequent *in vitro* studies showed that anaerobic bacteria, such as GV, metabolized TVF to a greater extent when compared to lactobacilli spp. (Klatt *et al.*, 2017). This raises the question of whether dysbiotic bacteria present in a non-lactobacilli dominant FGT could influence the efficacy of ARVs used for PrEP in young women.

Current diagnosis consists of the Nugent Score and Amsel Criteria. The Nugent Score is determined after a Gram stain of the FGT fluid of a patient with suspected BV. This is done to differentiate between healthy lactobacilli, small gram-variable bacteria and curved gram-variable bacteria that could be indicative of BV infection. A score of 7-10 is indicative of BV (Petrova *et al*., 2017). The Amsel Criteria is a list of symptoms used for diagnosis; thin, white or yellow homogenous discharge; presence of Clue cells; a vaginal fluid pH of >4.5 and a fishy odor upon addition of an alkali (Mohammadzadeh *et al*., 2015).

Table 1.2: Recent PrEP efficacy trials and their results (adapted from Romano *et al.*, 2013).

Study	**Product**	**Population**	**Efficacy result**	**References**
CAPRISA 004	1% TVF Gel	HIV-negative women	39%, p= 0.017	(Abdool Karim and Abdool Karim, 2010)
TDF2	Daily oral Truvada	HIV-negative women and men	63%, p = 0.013	(Thigpen *et al.*, 2012)
FEM PrEP	Daily oral Truvada	HIV-negative women	Early termination	(Van Der Straten *et al.*, 2012)
MTN 003 (VOICE)	Daily oral TVF; 1% TVF Gel	HIV-negative women	Early termination	(Chirenje *et al.*, 2010)

The FGT microbiota is dominated by lactobacilli strains such as *Lactobacillus. crispatus* and *Lactobacillus. jensenni.* These strains produce hydrogen peroxide and lactic acid to maintain an acidic microenvironment that protects the FGT against pathogenic viruses (O'Hanlon *et al.,* 2013). However, the microbiota associated with BV can be extremely complex which contributes to the difficulty in treating and preventing it. Unexpectedly, a study of Caucasian women showed that *L. crispatus* was the most dominant FGT bacterium in women without BV, and the outgrowth of *Lactobacillus. iners* was associated with BV (Verstraelen *et al*., 2009). Surprisingly, when women living in sub-Saharan Africa were analyzed, the FGT microbiota associated with BV was very different, not only from Caucasian women but also within groups of African women. For example, Pendharkar *et al.* (2013) found that South African women without BV had a FGT microbiome dominated by *L. crispatus* (Pendharkar *et al.*, 2013) whereas Dols *et al.* (2011) showed that South

African women who were HIV-negative and BV-negative had a *Lactobacillus. salivarius* dominated FGT microbiota (Dols *et al.*, 2011). Different combinations of lactobacilli spp. and some anaerobic bacteria were associated with both healthy and non-healthy FGT microbiotas amongst different groups of women. This emphasized how complex BV could be which complicates diagnosis and treatment.

1.8 BV-associated bacteria

Although Zhou *et al.* (2007) reported that African women were more likely to have BV compared to Caucasian women, the reason is still unknown. It has been suggested that ethnic groups might have different 'healthy' FGT microbiotas (Zhou *et al*., 2007). The role of bacterial species involved in BV remains controversial. According to Machado and Cerca (2015) there are two hypotheses regarding the etiology of BV: the single pathogen hypothesis and the polymicrobial hypothesis (Figure 1.5) (Machado and Cerca 2015). The single pathogen hypothesis suggests that one pathogenic bacterial species is responsible for causing BV (Danielsson *et al*., 2011) whereas the polymicrobial hypothesis speculates that BV is caused by a combination of anaerobic pathogenic bacteria (Romero *et al.*, 2014). Although women with BV have different combinations of bacteria, GV was found to be the dominant species present in most cases (Machado and Cerca 2015). Unlike *P. bivia,*

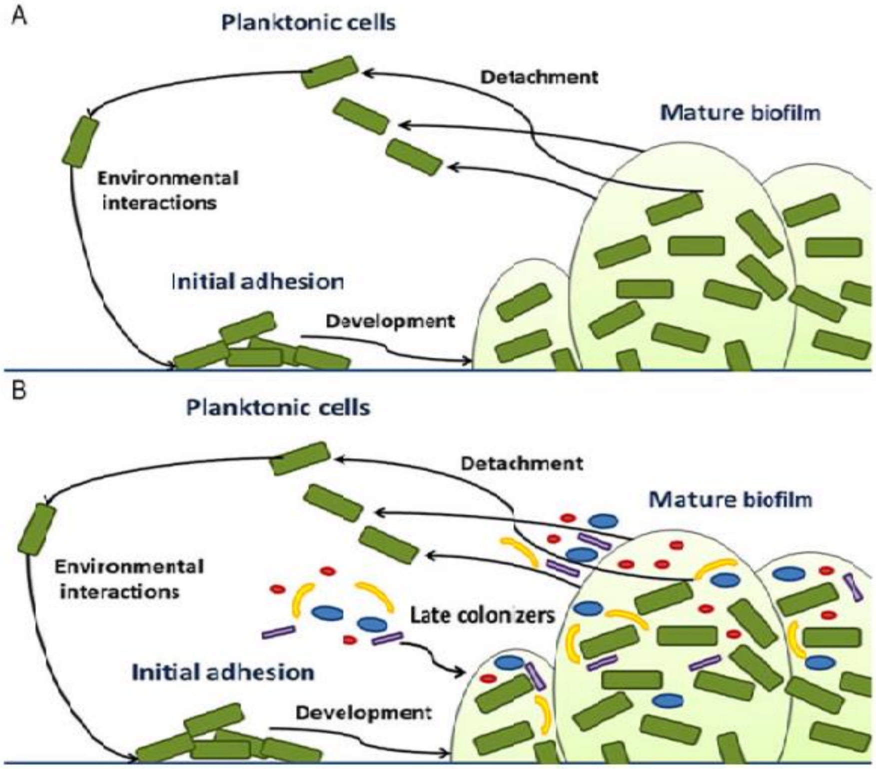

Figure 1.5: Diagram depicting single pathogen biofilm formation (A) and polymicrobial biofilm formation (B). (Machado and Cerca 2015)

Atopobium vaginae, Mobiluncus mulieris, and *Peptoniphilus* species, GV adheres to vaginal epithelial cells, forms biofilms and displays cytotoxic abilities (Patterson *et al*., 2010). These abilities and the formation of biofilm could allow for potential secondary colonizers, as shown in Figure 1.5. Secondary colonizers include *P. bivia*, which is known to have a synergistic and commensal relationship with GV (Randis and Ratner 2019). Studies have also shown that the biofilm produced by GV could stimulate the growth of *P. bivia* as well as other BV-associated bacteria (BVAB)(Datcu *et al*., 2013).

1.9 *Gardnerella vaginalis* diversity

The role of GV in BV has long been a topic of controversy and for many years it was hypothesized that GV was the sole culprit of BV (Catlin,1992). However, GV, a gram-variable anaerobe, was found in the FGT of both BV-positive and BV-negative women (Zozaya-Hinchliffe *et al.*, 2010) and Machado and Cerca (2015) found that GV was present in women with healthy vaginal microbiotas. Muzny *et al.* (2019) has proposed an alternative model where GV initiates the formation of a pathogenic, polymicrobial environment that results in BV. Genetic sequencing based on the chaperonin 60 universal target (cpn60) showed that GV was divided into 4 sub-groups, with the subgroups further classified into 4 clades A, B, C and D (Ahmed *et al.*, 2012; Schellenberg *et al.*, 2016). In 2019, 13 new species of the genus were found, including *Gardnerella. leopoldii*, *Gardnerella. swidsinskii,* and *Gardnerella. piotti* (Vaneechoutte *et al.*, 2019). These species correspond to three of the previously defined subgroups: *G. vaginalis* (subgroup C/clade 1), *G. leopoldii* and *G. swidsinskii* (subgroup A/clade 4), and *G. piotii* (subgroup B/clade 2) (Khan *et al.* (2019)). The high diversity of *Gardnerella* suggested a third model: phenotypic variation across sub-groups and/or strains, rather than just the presence of the bacteria itself, could drive the onset of BV. Janulaitiene *et al.* (2017) conducted a study to identify which clades of GV were most associated with BV. It was found that the FGT of 87% of the participants were colonized with GV but were BV-negative, with clade 4 (subgroup A) being the most frequently detected (Janulaitiene *et al.*, 2017). Further analysis showed that clades 1 and 2 (subgroups B and C) were significantly more present in participants who had a Nugent score of 7-10 (BV-positive).

1.10 Virulence determinants: Sialidase, Biofilms and Vaginolysin

Sialidase is a neuraminidase that cleaves terminal sialic acids from glycoproteins and glycolipids found in the mucosal-rich FGT. Sialidases can be categorized as either exo-sialidases or endo-sialidases, however the latter are mostly found in viruses and some strains of *Escherichia coli* (Juge *et al.,* 2016). Exo-sialidases can be further classified as hydrolytic, trans-sialidases and IT-sialidase based on their substrate-specificity and catalytic mechanisms (Juge *et al.,* 2016). Three genes are known to code for three different sialidases produced by GV, namely *nanh1, nanh2* and *nanh3*. Robinson *et al.* (2019) found that *nanh2* and *nanh3* were found in GV isolates that were sialidase positive and that NanH2 and NanH3 were the primary sources of sialidase activity in GV that led to BV pathology (Robinson *et al.*, 2019).

This enzymatic activity could explain how GV could influence the onset of BV (Lewis *et al.*, 2013). Soong *et al.* (2006) showed in the context of respiratory infections, that when the sialidase gene of *Pseudomonas aeruginosa* was knocked out, the bacterium failed to establish mucosal cell infection. Additionally, the sialic acid products could act as nutrients for surrounding bacteria, allowing for the establishment of biofilms (Thomas *et al.*, 2007). Interestingly, a study conducted by Moncla *et al.* (2016) found that sialidase activity was significantly higher in women who had a high Nugent Score (BV-positive) compared to women with intermediate or low Nugent scores and was used to diagnose BV (Moncla *et al.*, 2016). It has also been found that sialidase produced extracellularly by GV hydrolyzed mucosal sialoglycans in the FGT, both *in vitro* and *in vivo*, and that women who were BV-positive had a significant depletion in mucosal sialic acid (Lewis *et al.*, 2013). This indicates that GV, specifically the strains that produce sialidase, could be responsible for the pathology of BV and potentially other subsequent infections (Janulaitiene *et al.*, 2017).

The pathology of BV can also be due to the ability of GV to form biofilms in the FGT. Biofilms are when groups of microorganisms form a dense matrix on a surface consisting of polysaccharides, nutrients and proteins (Høiby *et al.*, 2011). Alves *et al.* (2014) found that GV had the greatest biofilm forming abilities compared to 29 other BVAB, and that GV initiates the polymicrobial environment (Alves *et al.*, 2014), driving the formation of nutrient-rich scaffolds that allow BVAB to flourish (Verstraelen and Swidsinski, 2013). In addition, the formation of biofilms by bacteria allows for evasion of the host immune system (Xie *et al.*, 2012). Therefore, GV and its ability to form biofilms could allow for BVAB to go undetected ensuring dysbiosis and hence BV. Biofilm formation has also been shown to impact the treatment efficacy of oral metronidazole, leading to high reoccurrence of BV (Swidsinski *et al.*, 2008). Therefore, GV and biofilm formation could be responsible for BV pathology.

Other than sialidase and biofilm formation, GV was found to produce a cytolytic toxin that impacted erythrocytes, nucleated cells, human endothelial cells and neutrophils (Rottini *et al.*, 1990). Vaginolysin (VLY) is a pore-forming toxin (PFT) and is a member of the cholesterol-dependent cytolysin (CDC) family (Gelber *et al.*, 2008). These proteins are dependent on high levels of cholesterol on the surface of target cells and when bound, undergo major conformational changes to form membrane-spanning β-hairpins that puncture cellular membranes, ultimately killing them (Christie *et al.*, 2018). VLY functions as a hemolysin, disrupting the protective mucosal layer and facilitating the colonization of BVAB (Gelber *et al.*, 2008).

1.11 Diversity linked to virulence

However, as seen with sialidase production, not all strains of GV produce VLY (Janulaitiene *et al.*, 2017). Looking at 14 isolates of GV, Janulaitiene *et al.* (2017) investigated the ability of these isolates to form biofilms as well as produce VLY and sialidase. There was no statistical difference in the production of VLY amongst any of the isolates (Janulaitiene *et al.*, 2017). Interestingly, Shishpal *et al.* (2020) found that the ATCC 14019 strain of GV had the ability to produce membrane vesicles containing VLY. Once these vesicles were internalized by vaginal epithelial cells, they were cytotoxic (Shishpal *et al.*, 2020). With regards to sialidase production, Schellenberg *et al.* (2016) characterized GV based on cpn60 and determined that only subgroups B and C had significant sialidase activity (Schellenberg *et al.*, 2016), suggesting that the association of subgroups B and C with BV could be due to the expression of NanH2 /or NanH3. However, focusing on specific strains and the presence or absence of individual virulence factors may not be sufficient to explain the impact GV has on the FGT and subsequently BV. It is possible that the synergistic presence of more than one strain of GV or other BVAB and their arsenal of virulent factors are needed to ensure dysbiosis of the FGT microbiome. It is also possible that strains are ecologically distinct and environmental variation impacts the virulent factors produced, especially in the presence of other complex, anaerobic bacteria. Another interesting speculation is that GV could produce a yet unidentified virulence factor that enables the onset of BV. Irrespective of the mechanism, the presence of BV, and potentially the presence of GV, seems to increase susceptibility to other infections, such as HIV.

1.12 BV and HIV

Bacterial Vaginosis is associated with increased HIV infection (Atashili *et al.*, 2008) and it has been suggested that inflammation associated with BV could recruit HIV target cells to the FGT, disrupt the vaginal epithelial barriers and wound-healing repair mechanisms (Klatt *et al.*, 2017). In relation to ARV efficacy, TVF levels were lower in women with non-lactobacilli dominated FGTs.

This finding was significantly associated with an increased abundance of *P. bivia* in these women (p= 0.023). However, TVF levels were rapidly degraded by GV, suggesting that this BVAB could be responsible for the decreased efficacy of ARVs in women with BV (Klatt *et al.*, 2017).

Taneva *et al.* (2018) reported that GV when exposed to TVF, produced adenine extracellularly, which was found to inhibit endocytic uptake of TVF by Jurkat cells (Taneva *et al.*, 2018). However, Klatt *et al.* (2017) showed that TVF was metabolized to adenine resulting in an increase intracellularly (Klatt *et al.*, 2017). Despite the difference in mechanism, both studies suggest that GV might prevent the uptake of ARVs by cells, depleting the concentration required in the FGT to protect against HIV-1 infection. Interestingly, Taneva *et al.* (2018) also showed that *L. crispatus* took up TVF albeit through an alternative mechanism. This was contrary to Klatt *et al.* (2017) and more recently, (Cheu *et al.*, 2020), both of whom showed that *Lactobacillus* did not take up ARVs. The variation between studies was suggested to be due to differences in bacterial cell culture conditions and preparation of GV samples (Taneva *et al.*, 2018). It is also possible that uptake and metabolism of ARVs is differentially affected because of variation between strains of *L. crispatus* and GV. Therefore, mere identification of *Gardnerella* spp. and lactobacilli spp. in the FGT might not be sufficient indication of increased risk of HIV acquisition but that a more strain-specific approach is required.

Furthermore, this is further complicated by the global variation in microbiota associated with BV as reviewed by Woodman (2016), The FGT of BV-positive women from sub-Saharan Africa and of African American heritage is colonized by different bacterial species compared to Caucasian cohorts. This is likely due to numerous factors such as host genetics, immune system and the environment they live in (Woodman,2016).

Unexpectedly, unpublished preliminary data indicated that a strain of GV that does not produce sialidase inhibited HIV-1 entry of a human reporter cell line. This surprising finding suggests that certain strains of GV might play a protective role against HIV-1 infection, either through the production of proteins or secondary bioactive metabolites. GV is present in most women (with or without BV) (Pleckaityte *et al.*, 2012) and despite not knowing the full extent of its diversity or virulence, this potential protective mechanism warrants further investigation.

Therefore, identifying and investigating the strains of GV present in women with BV might be required to diagnose women with BV. In addition, understanding the full biochemical potential of these strains could aid in the identification of better HIV preventative treatments for women. Taha *et al.* (1998) suggested that treating women for BV could restore normal FGT flora, thereby reducing susceptibility to HIV infection (Taha *et al.*, 1998).

1.13 Natural Products

Natural products (NPs) are chemical compounds isolated from diverse living things. These products could be the result of primary metabolism, directly required and used by the organism, or secondary metabolism, which are used by the organism to adapt to different environmental stimuli (Singh,R *et al.*, 2017). Primary products include polysaccharides, nucleic acids, and fatty acids, whereas secondary products are more diverse with obscure functions. Secondary metabolites that exhibit biological functions are deemed bioactive secondary microbial metabolites. These metabolites display unique chemical structures and have versatile bioactivities (Demain,1999). Today, these metabolites have been redefined as products that regulate growth processes, replications and/or exhibit some kind of effect to the life cycle of prokaryotic and eukaryotic cells at the biochemical level (Bérdy,2005).

Bioactive secondary microbial metabolites have been utilized for many centuries, with products being found as early as 350-550 CE (Bassett *et al.*, 1980). One of the greatest class of secondary metabolites discovered were antibiotics, specifically penicillin in 1928, which drastically changed therapeutic medicine and infectious disease research (Bennett and Chung 2001). With the increase in technological advancements, the 1940s saw the Waksman group at Rutgers University discover antibiotics such as Actinomycin D, Streptomycin and Neomycin produced by the filamentous actinobacteria, actinomycetes (Demain,2014). Continuous research conducted by numerous groups saw the expansion of antibiotics and useful NPs being discovered. It then became clear that actinomycetes were “metabolically talented” with the *Streptomyces* genus being the most noteworthy producers due to the sheer number, versatility and diverse structures of metabolites produced (Ludwig *et al.*, 2012). Similarly, metabolites showing antifungal (kasugamycin), anti-viral (hygromycin) and antitumor (doxorubicin/andriamycin) properties were also discovered, broadening the range of NPs that could be used against diseases affecting humans, animals and agriculture (Barka *et al.*, 2016). However, over time, attention turned to rare filamentous actinobacteria (or non-*Streptomyces* actinobacteria) which produce 25-30% of all antibiotics (Ding *et al.*, 2019).

Despite the advances made with using NPs as drugs, there are challenges faced. One of the most important considerations is determining the safety of the drug, the "maximum tolerated dose" in clinical trials (Mathijssen *et al.,* 2014) or the therapeutic index (Figure 1.6). Identifying the range of concentrations that are therapeutic and not toxic is a vital prerequisite for drug discovery.

Furthermore, the process of identifying, testing an identified compound on a bioassay and isolating it can be extremely tedious and slow. In addition, NPs have diverse, complex structures making it difficult to determine the complete structure of compounds as well as replicate it for large-scale production. For large-scale production of antimicrobials, an extensive source of

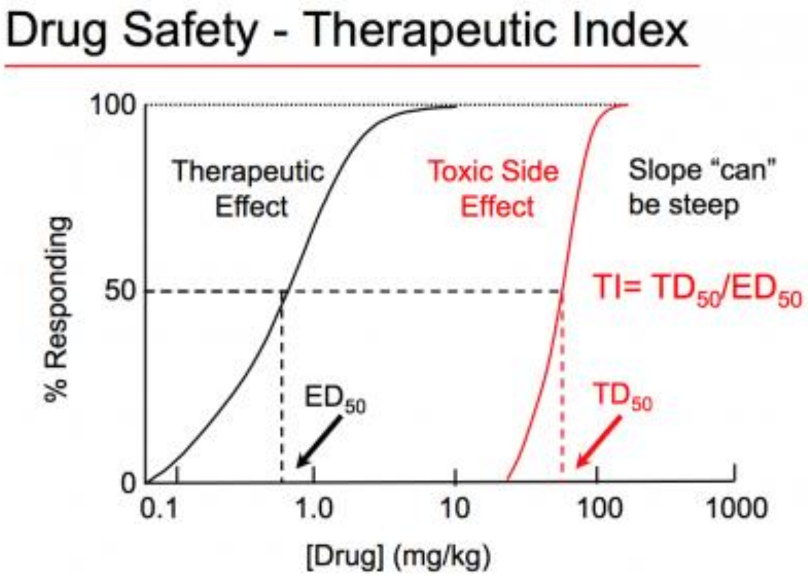

Figure 1.6. Using the Therapeutic index to determine drug safety. Drugs over a range of concentrations have therapeutic effects, toxic side effects, and in some cases lethal effects. Cytotoxicity is typically dose-dependent and can be determined by identifying the drug concentration that produces a toxic effect in 50% of the population (TD_{50}) and (in animal models) lethal effects in 50% of the population (LD_{50}). It is possible that dose-relationships for cytotoxicity may have different slopes compared to the therapeutic dose-response relationship because they have different mechanisms of action such acting via different receptors. (Adapted from https://tmedweb.tulane.edu/pharmwiki/doku.php/basic_principles_of_pharm).

bacteria would be required which could come with an additional challenge if the bacteria live in extreme, remote and inaccessible locations. (Beutler,2010). However, advances in sequencing technology as well as databases such as the Global Natural Products Social Molecular Networking (GNPS) greatly assist in the identification of unknown compounds found in natural products and play a pivotal role in the subsequent research conducted on these potentially useful compounds. Identifying these compounds in *Actinomycetota*, a phylum famous for producing thousands of secondary bioactive metabolites, could lead to the discovery of novel treatments, especially in the context of HIV and BV. Additionally, drug repurposing could lead to novel treatments. Drug repurposing is the concept of using known drugs, for example antibiotics, to investigate their potential to target other microorganisms, such as viruses (Talevi and Bellera, 2020). For example, it has been shown that ciprofloxacin and moxifloxacin showed antiviral activity against SARS-CoV-2 by binding to the Main Protease, binding to the protein's active site at a stronger efficacy than the natural ligand (Marciniec *et al.*, 2020). This concept could be beneficial in the search for more effective treatments for women vulnerable to HIV infection.

1.14 Actinobacteria

Actinobacteria are free-living organisms found in both terrestrial and aquatic environments (Ludwig *et al.*, 2012). Classified as Gram-positive, filamentous and containing a high G-C content, this class is extremely relevant to human health, agriculture and biotechnology (Ventura *et al.*, 2007). They are known to grow communally with other bacteria, such as *Bacteriodes* and *Staphylococcus* and are found in the mouth, gut, and genital tract (Prieto-Granada, C. N. *et al.*, 2010).

Actinomycetota are one of the largest taxonomic units among 18 major lineages within the *Bacteria* domain. It consists of 5 subclasses, 6 orders and 14 suborders (Ludwig *et al.*, 2012). Actinobacteria are widely diverse not only in morphology, but also in physiology and metabolic capabilities. The phylum is delineated based on 16S rRNA, however this can cause ambiguity between closely related species (Ōmura *et al.*, 1982). Therefore, additional genetic markers such as *rpoB* and *ssgB* are used for more closely related species (Traag *et al.*, 2013). The phylum is divided into 6 classes: *Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria,* *Rubrobacteria,* and *Thermoleophilia.* Thereafter, the class *Actinobacteria* is made up of 16 orders including *Actinomycetales* and *Bifidobacteriales* (Zhi *et al.*, 2009). The order *Actinomycetales* is now a restricted member of the family *Actinomycetaceae* (Zhao *et al.*, 2014)

The discovery of antibiotics, antifungals, and antitumor NPs from filamentous bacteria have made large strides in the medical and environmental fields. With pressing issues such as HIV infection, ARV drug resistance and the association between BV and HIV there is a need to focus on the identification of novel, anti-viral NPs for the treatment and prevention of HIV-1 infection (Ma *et al.,* 2020). NPs with anti-viral properties have recently been utilized and found effective against certain diseases. For example, it has been found that shikimic acid, an organic compound found in plants and microorganisms which plays an important role in the production of aromatic amino acids and various alkaloids, is the primary progenitor of the anti-influenza drug oseltamivir (sold as Tamiflu) (Singh, P *et al.*, 2019). It inhibits influenza A and B sialidase, preventing the virus budding from the host cell, and stopping viral replication (Singh, P *et al.*, 2019). Another example is the production of avermectin by *Streptomyces avermitilis,* which later led to the FDA-approved drug Ivermectin (Omura *et al.*, 2001). Ivermectin is an antiparasitic drug that displays a wide range of anti-viral capabilities against numerous viruses *in vitro*, such as the Dengue virus (Wagstaff *et al.*, 2011), West Nile virus (Yang *et al.*, 2020), Venezuelan equine encephalitis virus (Lundberg *et al.*, 2013) and influenza (Gordon *et al.*, 2020). Interestingly, Ivermectin was originally identified as an inhibitor of HIV-1 Int and its interaction with the importin α/β1 heterodimer which is responsible for Int nuclear transport, ultimately inhibiting Int nuclear import and viral replication (Wagstaff *et al.*, 2011).

Studies such as these highlighted the diversity and complexity of NPs and their potential as a source of anti-viral products (Ma *et al.,* 2020). A study conducted by Chen *et al.* (2018) also highlighted the extraordinary ability of filamentous actinobacteria to produce anti-HIV-1 NPs. This study identified the production of 9 compounds from *Streptomyces* sp. CPCC 202950 and found that compounds 1,2 and 4-9 (Figure 1.7) could inhibit HIV-1 protease. Compound 1 was identified as Ahmpatinin ^{i}Bu and contained an unusual amino acid, 4-amino-3-hydroxy-5-(4-methoxyphenyl) pentanoic acid. Interestingly, Ahmpatinin ^{i}Bu exhibited significant inhibitor activity against HIV-1 protease with an IC_{50} value of 1.79 nM (Chen *et al.*, 2018).

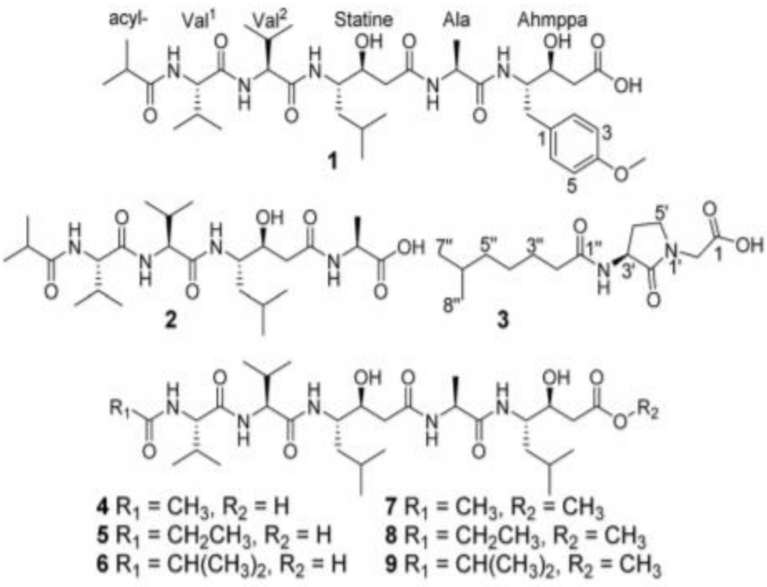

Figure 1.7: Chemical structures of compounds 1,2 and 4-9 produced by *Streptomyces* sp. CPCC202950 that inhibited HIV-1 protease (Chen *et al.,* 2018)

Similarly, a study by Xiao Liu *et al.* (2020) extracted and identified a pentapeptide, 4862F (Figure 1.8), from *Streptomyces albosporus* 103A-04862 that displayed inhibitory activity against HIV-1 protease (Liu *et al.*, 2013). Another study by Shao *et al.* (2020) reported that berberine, an isoquinoline quaternary alkaloid derived from medicinal plants that is used to treat diarrhea, bacillary dysentery and gastroenteritis, is effective in inhibiting HIV-1 entry with an IC_{50} range of 6.36 ±0.34 µg/mL. It does so by undergoing a conformational change, forming a novel NHR-berberine-CHR complex with gp41 , preventing fusion of HIV with the host cell (Shao *et al.*, 2020). Although this was not derived from filamentous actinobacteria, it shows the potential in screening for natural products and testing their capabilities against HIV-1 infection.

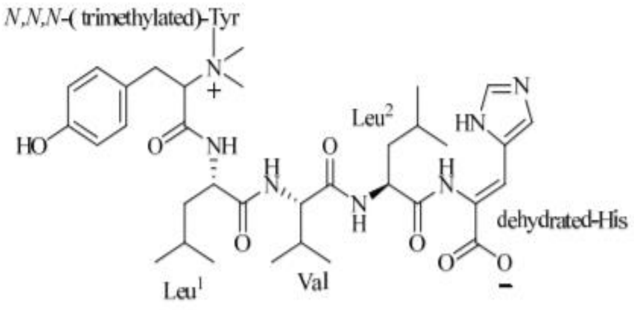

Figure 1.8: Chemical structure of pentapeptide 4862F produced by *Streptomyces albosporous* 103A-04862 that showed inhibitory activity against HIV-1 protease (Liu *et al*., 2013)

1.15 BV and Natural Products

The FGT microbial population is highly diverse and might be differentially associated with BV and HIV infection due to variation in pathogenicity (Pleckaityte *et al.*, 2012). For example, sialidase and VLY produced by only some GV strains might provide a growth advantage that facilitates the displacement of protective lactobacilli spp. and hence onset of BV (Castro *et al.*, 2013). These virulence factors might also facilitate HIV infection by enhancing endocytosis, decreasing viscosity of FGT mucosa and enhance binding of HIV to target cells (Cohen *et al.*, 2012).

Because of the association between BV and increased HIV acquisition, and the evidence suggesting that GV plays an important role in BV etiology, we could speculate that GV directly enhances HIV infection. It has been shown that bacterial sialidase increases HIV infection (Sun *et al.*, 2001), suggesting that GV sialidase may have a similar effect. However, HIV infection of macrophages is facilitated by the interaction between gp120 sialic acids and sialic acid-binding immunoglobulin-like lectins (Siglec) and the removal of the sialic acids led to a decrease in HIV infection of macrophages (Zou *et al.*, 2011). We found that the supernatant of GV inhibited the infection of HIV without cytotoxicity (Unterpretinger and Abrahams, unpublished data) but as this strain did not express sialidase, it is possible that when the bacteria are grown under laboratory conditions, they produce compounds that may have other anti-viral activity. This suggests that GV may play a role directly in influencing HIV infection in the FGT and thus provide targets for the design of novel therapeutics or they may be a source of ARVs.

Taneva *et al.* (2018) showed that adenine, a metabolite of TVF breakdown impacted HIV infection of Jurkat cells, suggesting that further study could identify alternative NPs (Taneva *et al.*, 2018). These products may be secondary metabolites or proteins that differentially affect BV onset, HIV infection and the efficacy of ARVs according to species or strains. Therefore, screening these microorganisms may identify novel NPs specific to some strains of GV so that their introduction and colonization of the FGT could stave off HIV infection.

1.16 Rationale

HIV-1 is a pandemic affecting the lives of many people, especially young, vulnerable heterosexual women in Sub-Saharan Africa. Factors such as HIV-1 drug resistance, drug-drug interactions, pharmacokinetics, sexually transmitted diseases, adherence and BV have reduced treatment efficacy. Natural products have shown great diversity, extensive versatility and have greatly contributed to modern medicine. Such advances have increased human life span and led to effective treatments for numerous life-threatening diseases. In more recent years there has been a shift of focus from antibiotics and antifungal NPs to those displaying anti-viral properties. In the context of BV and HIV, screening filamentous actinobacteria and BVAB for novel anti-viral NPs could aid in the design of effective treatments for women suffering from BV and who are at high risk of HIV-1 infection. GV has been at the forefront of understanding the etiology of BV since the 1950s, with current research showing the increasing complexity and diversity of the genus, *Gardnerella.* GV has been shown to display an arsenal of virulence factors, such as sialidase, VLY and biofilm formation, that are associated with BV and play a role in enhancing HIV-1 infection. GV is also present in women who are BV-negative and surprisingly, it has been found that a commercial strain of GV may play a protective role against HIV-1 infection (Alessandra Unterpertinger and Bianca Abrahams, unpublished data), emphasizing the need to further investigate the encompassing role GV plays in the FGT and how one can harness its abilities to find effective treatment for women vulnerable to both BV and HIV-1 infection.

Therefore, this study aims to identify novel anti-viral compounds from filamentous actinobacteria and *G. vaginalis* that impact HIV-1 infection.

1.17 Aim:

The overall aim of this project is to investigate whether filamentous actinobacteria and *Gardnerella vaginalis* produce compounds which impact HIV-1 infection.

1.18 Objectives:

1. Determine whether filamentous actinobacteria produce active compounds which inhibit the single round infection of PSV using a reporter cell line.
2 Extract and identify, potential bacterial compounds produced by *G. vaginalis* that impact HIV infection.

Chapter 2: Materials and Methods

2.1 Biosafety Statement

This project was approved by the Faculty of Health Sciences Biological Safety Committee, University of Cape Town (ZW/001/2020-2023) for the use of hazardous biological agents and/or genetically modified organisms. The cell lines used were maintained and used under Biosafety Level II conditions, and experiments using HIV pseudovirus (PSV) were carried out under Biosafety Level II infrastructure in the department of Medical Biochemistry and Structural Biology, University of Cape Town. Bacterial culturing and maintenance of GV was conducted under Biosafety Level II conditions in the department of Medical Microbiology, University of Cape Town. The culturing of actinobacteria, downstream extraction processes and mass spectrometry was conducted under BSL I conditions in the Division of Clinical Pharmacology, Department of Medicine, University of Cape Town.

2.2 Cell lines

The adherent cell lines, Human embryonic kidney (HEK) 293T (ATCC) and TZM-bl cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc) (Platt *et al.*, 1998) were grown in Dulbecco modified Eagle high glucose medium (DMEM) (Sigma) with 10% *(v/v)* fetal bovine serum (Sigma) and 1 U/mL penicillin and 1 µg/mL Streptomycin (Sigma). TZM-bl cells are modified HeLa cells with CD4, CCR5 and CXCR4 on the cell surface and carries a luciferase gene under the control of the HIV promoter (LTR).

2.3 Plasmids

HIV Env was cloned into a mammalian expression vector, pTARGET (Promega) and pSG3Δenv plasmid comprising an HIV genome lacking a functional *env* gene was used to generate PSV (AIDS Reagent Programme, NIH). Plasmids were prepared by transforming chemically competent *Eschericia coli* JM109 cells (Promega) with plasmid DNA (100 pg). Briefly, DNA and cells were incubated for 25 – 30 minutes on ice before heat shock at 42°C for 45 seconds. The cells were placed on ice for 2 minutes before Luria broth (LB) (1% *w/v* tryptone, 0.5% *w/v* yeast extract, 1% *w/v* NaCl in distilled water) was added to a final volume of 1 mL before incubation at 37°C, with shaking. After 1 hour, the culture was centrifuged at 5000 rpm for 5 minutes, 900 µl supernatant removed, and the cell pellet resuspended in the remaining supernatant. Total cell mixture was spread onto Luria agar (LA) [LB, 1.5% *w/v* agar and 100 µg/mL carbenicillin disodium salt (Sigma Aldrich)] plates and incubated at 37°C overnight.

2.4 Plasmid DNA extraction

Luria Broth was prepared in 5 mL and 50 mL aliquots. Plasmid glycerol stocks of JM109 *E. coli* were prepared by autoclaving 500 µl of 50% (*v/v*) glycerol in a cryotube. Thereafter, 500 µl of culture were removed and placed in the cryotubes and flash frozen in liquid nitrogen, and stored at -80 °C. These stocks were used to inoculate LB supplemented with carbenicillin (Inqaba, AM J358-1G) (100 µg/mL). Starter cultures (5 mL) were grown at 30°C for approximately 17 hours, with shaking. The starter cultures were then transferred to 50 mL LB, supplemented with carbenicillin (100 µg/mL) and the same procedure was followed. DNA was extracted from 50 mL cultures using a Midiprep Kit (Qiagen, 12145) and the Plasmid DNA Purification kit (Qiagen) according to the manufacturer's specifications. DNA was then stored at -20°C until required.

2.5. Actinobacteria Cultivation and Extraction

Twenty-three strains of filamentous actinobacteria were acquired from the Meyer's culture collection from Dr Paul Meyers, Department of Molecular and Cell Biology, University of Cape Town. The strains of actinobacteria were cultured in #553 GPHF-MEDIUM (DSMZ) (German Culture Collection) or 174 Bennett's (1% *w/v* yeast extract, 1% *w/v* beef extract, 2% *w/v* N-Z Amine Type A, 10% *w/v* glucose, 1 L distilled water). The cultures were incubated for 10 days at 30°C and shaking at 100 rpm. Once grown, the purity of the cultures was initially tested by streaking to select a single culture followed by Gram stain (O'Toole, 2016). These media were selected as they are known to induce production of active antimicrobial agents in previous studies performed by Dr Daniel Watson. Once purity was confirmed by the identification of filamentous bacteria by Gram-stain, 5 mL of methanol and 30 mL of ethyl acetate were added to the culture and left to shake at 90 rpm for 1.5 hours. Thereafter, the culture was placed in a separating funnel for 20 minutes to allow the aqueous and organic phases to separate. After separation, the ethyl acetate layer was removed and left to dry in a beaker overnight in a fumehood. Once dried, the extracts were transferred to weighed Eppendorf centrifuge tubes in ethyl acetate, dried down and resuspended in 50% DMSO to produce final stock samples at a final concentration of 20 mg/mL.

2.6. *Gardnerella vaginalis*

Frozen glycerol stocks (25% *v/v*) of the ATCC14018 strain of GV were used to inoculate 15 mL aliquots of Brain Heart Infusion (BHI) (Sigma-Aldrich, #53286) media. The culture was then incubated anaerobically at 37°C for 48 hours using gas packs (ThermoFisher).

2.6.1 Confirmation of purity

2.6.1.1 16S rRNA PCR and sequencing

The bacterial cultures of GV strain ATCC14018 were mixed by vortexing before streaked on BHI agar plates to obtain single colonies. The plates were incubated anaerobically at 37^{o}C, 5% CO_2, for 48 – 72 hours. The plates were evaluated for contamination by determining a homogenous culture of small, round, sticky and cream-colored colonies. An isolated colony was picked and resuspended in 200 µL sterile 1x PBS (WhiteSci) with 0.1 mg/mL proteinase K (Sigma) and incubated at 45°C for 20-30 minutes. The samples were then heat-inactivated at 85°C for 20 minutes and centrifuged at 5000 rpm for 3 minutes in a benchtop centrifuge. The supernatant was collected for subsequent 16S rRNA PCR. The sequences of the primers are as follows:

F27 – AGAGTTTGATCMTGGCTCAG
R5 (1492R) - TACGGYTACCTTGTTACGACTT
For a 25 µL reaction, the following was added to a PCR tube:

Component	**Final concentration**	**1x reaction (µL)**
10 µM Forward primer	0.2 µM	2.5
10 µM Reverse primer	0.2 µM	2.5
10 mM dNTPs	0.2 mM	0.5
5X One*Taq* Standard reaction buffer*	1X	5
One*Taq* HotStart Polymerase	0.025 U/µL	0.125
DNA/Sample	10 ng/µL	3
Nuclease-free water	n/a	Add to 25 µL
Final volume		**25**

The samples were mixed and centrifuged on a benchtop centrifuge for 10-20 seconds at 5000 rpm. The PCR parameters were as follows:

94°C for 5 min

$$\left.\begin{array}{l}94^\circ\text{C for 30 sec}\\55^\circ\text{C for 30 sec}\\65^\circ\text{C for 90 sec}\end{array}\right\}\text{— X 30 cycles}$$

72°C for 7 min

The expected PCR product of approximately 1500 bp was identified using a 1% agarose gel before the product was sent to Central Analytic Facility, University of Stellenbosch for sequencing and a subsequent BLAST analysis was done (NCBI).

2.6.1.2 *Gardnerella vaginalis* specific PCR

Screening using 16S rRNA was a time-consuming process and so a GV specific PCR was designed to amplify species-specific regions of the 16s rRNA gene (Fredricks *et al.*, 2007).

GV Forward 5’-GGGCGGGCTAGAGTGCA-3’

GV Reverse 5’-GAACCCGTGGAATGGGCC-3’

For a 25 µL reaction, the following was added to a PCR tube:

Component	**Final concentration**	**1x reaction (µL)**
10µM Forward primer	0.2 µM	0.5
10µM Reverse primer	0.2 µM	0.5
10mM dNTPs	200 µM	0.5
10X Standard Taq buffer	1 x	2.5
Standard Taq (NEB)	1.25 units/50 µl PCR	0.125
DNA/Sample	n/a	2-4
Nuclease-free water	n/a	Add to 25 µL
Final volume		**25**

The samples were mixed and spun down for 10-20 seconds at 5000 rpm on a benchtop centrifuge. The PCR parameters were as follows:

95°C for 30 sec

$$\left.\begin{array}{l}95^{\circ}\text{C for 30 sec}\\ 62^{\circ}\text{C for 45 sec}\\ 68^{\circ}\text{C for 15 sec}\end{array}\right\}\text{ X 30 cycles}$$

68°C for 5 min

The 210 bp PCR product was visualized using gel electrophoresis at 70 V for 60-90 min using a 1.5% agarose gel (WhiteSci).

2.6.2. Enrichment and Extraction of active fractions

2.6.2.1 Liquid-Liquid extraction

Liquid-liquid extraction (LLE) was used to isolate organic small molecules from GV cells and culture medium to identify molecules that impact PSV infection. GV ATCC14018 was inoculated into 15 mL or 50 mL BHI and grown anaerobically for 48 hours at 37°C or until turbid. The culture was centrifuged for 5 minutes at 1200 RCF (3125 rpm) and the supernatant removed. Equal volumes of ethyl acetate and culture supernatant were poured into a separation funnel and the mixture allowed to separate for 30 minutes at room temperature (20-25 °C). Once separated, the broth layer was transferred to a beaker. The separation process was repeated once more, and the broth fractions were pooled and dried down using nitrogen gas in the fume hood. The ethyl acetate layer was placed in a beaker for drying. The cell pellet was washed with methanol on a shaking incubator at 90 rpm for 20 minutes at room temperature (20-25 °C) and then centrifuged for 5 minutes at 1200 RCF. The supernatant was then dried down. This process was repeated with ethyl acetate and hexane until the following fractions were collected: Ethyl acetate broth, Methanol Cell Mass, Ethyl acetate Cell Mass, and Hexane Cell Mass. The fractions were then resuspended in 1.5 mL of their respective solvents and transferred to weighed Eppendorf centrifuge tubes to obtain final concentration of 20 mg/mL. The extracts were then tested for PSV inhibition using a cell-line based entry assay. To ensure that the resuspension solvent did not affect the PSV infection assay and/or cell viability, 0.05% and 0.1% DMSO and methanol were added to the infection assay and changes in luminescence were measured.

2.6.2.2. Solid Phase Extraction

Solid phase extraction (SPE) was used to further fractionate the LLE fractions that impacted PSV entry. A C18 column was equilibrated with 2 mL of hexane followed by 2mL of methanol, and the active sample was dissolved in a solution of 90:10 methanol: hexane and applied to the cartridge under pressure. The cartridge was then washed with 2 mL methanol, 2 mL acetonitrile and finally 2 mL hexane. The fractions were then dried down, resuspended in 1.5 mL of their respective solvent, and transferred to weighed Eppendorf centrifuge tubes before being dried down.

2.6.3. Ammonium Sulphate Precipitation

GV ATCC14018 was grown in 15 mL aliquots of BHI for 48 hours at 37°C, and then transferred to 35 mL aliquots and cultured until an OD=0.6 at 600 nm. The culture was centrifuged at 11294 rpm for 10 minutes at 4°C and the cell pellet was discarded. An aliquot of the initial culture was removed. The remaining culture medium was transferred to a beaker and Ammonium Sulphate (Sigma) was added to a final concentration of 10% (*w/v*) stirring constantly at 4°C and left on ice for 20 minutes. Thereafter, the solution was centrifuged for 10 minutes at 11294 rpm at 4°C. Once centrifuged, the supernatant was decanted into a new beaker and the process was repeated to achieve the range of concentrations . After centrifugation, the pellets were resuspended in 10 mL resuspension buffer (20 mM Tris, 50 mM NaCl at pH 8) and filter sterilized using a 0.2 µm filter into a 15 mL falcon tube.

To sequentially increase the concentration of AS to final concentrations of 20, 30, 40, 50, 60, 70, and 80% AS was added to the solution according to the following formula:

$G = (533 (S2 – S1) / (100 - 0.3 S1)$

Where:

S1 = % saturation in starting solution

S2 = % saturation in the final solution

G = grams of solid AS to be added per liter

2.6.3.1 Dialysis

After AS precipitation, the fractions underwent dialysis using a cellulose membrane (D9277-100FT, Merck) to desalt the samples. Each fraction was transferred to the dialysis tubing and left for 6 hours in 1 L of distilled water, stirring at 4°C. Thereafter, the water was replaced, and the samples left overnight stirring at 4°C. After dialysis, the samples were removed from the membranes and aliquoted and stored for future testing at 4°C. To concentrate the samples each fraction was freeze-dried using the VirTis Sentry^{TM} Freeze Drier and pellets were resuspended in 200 µL PBS and stored at -20°C.

2.7. Bicinchoninic acid protein assay

The protein concentration of samples was calculated using the Pierce™ BCA Protein Assay Kit (Catalog Numbers 23225 and 23227). Standards of Bovine Serine Albumin Standard (BSA) were prepared (Pierce ^{TM} BCA Protein Assay Kit), and the protein concentrations were calculated from the generated standard curve.

2.8. SDS-PAGE

An amount of one hundred µg protein was added to SDS 5 x loading buffer (5% *w/v* SDS, 20% *w/v* glycerol, 0.1% *v/v* β-mercaptoethanol and 0.1% *v/v* bromophenol blue) and heated at 90°C for 10 minutes to denature the proteins. A low concentration of acrylamide was used to confirm the presence of total protein in each sample and therefore, protein samples were loaded into a 5% acrylamide stacking gel and an 8% acrylamide resolving gel and run in 1X running buffer (0.025M Tris, 0.192M Glycine, 0.1 % SDS, pH=8.3) at 25 mA (100 volts) for 90 minutes using the Modular vertical gel system [Protean Tetra (BIO-RAD) < 10 samples/gel) and omniPAGE (Cleaver scientific, AEC Amersham) for >10 samples/gel]. The gels were then stained with Coomassie stain (50% *v/v* Methanol, 10% *v/v* Acetic acid, 1% *w/v* Coomassie Brilliant Blue) for 1 hour at room temperature (20-25 °C) and then treated with destaining solution (25% *v/v* Ethanol, 10% *v/v* Acetic acid). The gels were visualized on a light box.

2.9. Tissue Culture

HEK293T and TZM-bl cells were grown in DMEM (Sigma) which was supplemented with 10% *w/v* FBS (Sigma) and 1 U/mL penicillin and 1 µg/mL streptomycin (Sigma). Cells were incubated at 37°C and 5% CO_2 in a water-jacketed incubator.

2.9.1. Pseudovirus production in HEK293T cells

PSVs are viral particles restricted to a single round of infection due to an inactive *env* gene. These are produced by cloning the *env*-pseudotyped HIV-1 in HEK293T cells and co-transfecting with an Env-expressing plasmid and a HIV genome plasmid lacking a functional *env* gene. Co-transfection produces PSV particles that can infect cells but, are unable to produce infectious progeny. This round of infection is readily detectable in genetically engineered cell lines that contain a Tat-responsive reporter gene, specifically luciferase. HEK293T cells were seeded at a density of 2 x 10^6 cells per mL in 2 mL in a 6-well plate and incubated overnight at 5% CO_2 and 37°C. Cells were evaluated to ensure that approximately 40 – 60% were confluent before transfection. Transfection complexes were generated by adding DNA and polyethyleneimine (PEI) in a 3:1 ratio (2.5 µg Env plasmid + 5 µg of pSG3Δenv + 22.5 µL PEI), to 400 µL of serum-free medium.

Transfection mixes were vortexed for 15 seconds and incubated at room temperature (20-25 °C) for 10 minutes. Medium was removed from each well and replaced with 1.5 mL of fresh medium before complexes were added in a drop-wise manner. Cells were incubated for 48 hours, thereafter the PSVs were harvested from the 2 mL of culture medium from each well and filter sterilised using 0.2 µm filters. Thereafter, FBS was added to each sample to obtain a final concentration of 20% *w/v* FBS. The medium was mixed thoroughly and aliquoted into cryovials for long-term storage at -80°C.

2.10. Titration of Pseudovirus in TZM-bl cells

TZM-bl cells were plated in 96-well plates at a density of 50,000 cells per well and incubated overnight at 37°C and 5% CO_2. PSV was allowed to thaw at room temperature and then serially diluted in a deep-well plate. Concentrations of p24 were as follows: 100 ng/mL, 50 ng/mL, 25 ng/mL and 12.5 ng/mL.

One hundred µL of medium was removed from the well and replaced with 100 µL of each PSV diluted sample in quadruplicate. The cells were incubated for 48 hours. For cell lysis, 150 µL of medium was removed from each well and 50 µL of BrightGlo (Promega) was added to each well and incubated for 2 minutes. The wells were then mixed thoroughly through pipetting and 75 µL was transferred to an opaque 96-well plate before reading in the luminometer (Glomax® 96 Modulus Microplate). $TCID_{50}$ (Median Tissue Culture Infectious Dose) was determined by calculating the percentage of infected wells for each dilution and 200 $TCID_{50}$ was used in all infection experiments.

2.11 PSV entry assay

TZM-bl cells were plated in 96-well plates at a density of 50,000 cells per well and incubated overnight at 37°C and 5% CO_2. Two hundred µL of media was then removed. DEAE-Dextran (Sigma) was diluted into DMEM to a final concentration of 20.8 µg/mL and 100 µL of DEAE-Dextran DMEM was added to the wells. Thereafter, 50 µL of each sample was added to the well. Depending on the experiment, the sample was either the GV supernatant, to test its impact on

PSV entry, or the extracts of the filamentous actinobacteria strains. The PSV was diluted to 200 $TCID_{50}$ and 50 µL was added to each well. After incubation for 48 hrs at 37°C and 5% CO_2 , 150 µL of medium was removed from each well and 50 µL of BrightGlo (Promega) was added to each well and incubated for 2 minutes. The wells were then mixed thoroughly through pipetting and 75 µL was transferred to an opaque 96-well plate before reading in the luminometer (Glomax® 96 Modulus Microplate). TZM-bl are a HeLa cell derivative engineered by amphotropic retroviral transduction to express CD4 and CCR5. It was also engineered with an HIV-1-based vector to contain Tat-responsive reporter genes for firefly luciferase (Luc). Therefore, the entry of PSVs can be measured based on the amount of luciferase produced from the well.

2.12 MTT assay

TZM-bls were plated in 96-well plates at a density of 50,000 cells per well and incubated overnight at 37°C and at 5% CO_2. After a 48-hour incubation period, 10 µl of the MTT (Merck) labeling reagent (final concentration 0.5 mg/mL) was added to each well. The plate was incubated for 4 hours at 37°C, 5% CO_2. Thereafter, 100 µl of the solubilization solution (dimethyl sulfoxide or sodium dodecyl sulfate in diluted hydrochloric acid) was added into each well and the plate incubated overnight at 37°C, 5% CO_2. The plate was then evaluated for complete solubilization of the purple formazan crystals and the absorbance of the samples measured using microplate luminometer reader (Glomax® 96 Modulus Microplate). The wavelength to measure absorbance of the formazan product was between 550 and 600 nm.

2.13 Mass spectrometry and Global Natural Products Social Molecular Networking (GNPS)

All active samples, as well as controls, were diluted 100-fold in methanol to a volume of 200 µL for high pressure liquid chromatography-mass spectrometry using a quadrupole time-of-flight instrument (QTOF). Ten µL of the methanol-diluted sample was injected on to the C18 HPLC column (Agilent InfinityLab Poroshell 120 EC-C18, 4.6 mm X 150 mm, 4 µM). The aqueous mobile phase was water with 1mM ammonium formate, while the organic phase was methanol with 0.05% *v/v* formic acid. A gradient method was used that ran from 2% to 98% organic solution for

25 minutes, which was then held at 98% organic for a further 2 minutes before returning to 2% organic to equilibrate for 3 minutes before the next run. A blank sample was run between each sample to prevent carry over. HPLC-separated samples were then detected using an AB Sciex® X500R QTOF mass spectrometer to obtain MS1 and MS2 data using information dependent acquisition (IDA), within a range of 50-1500 Da. Raw high resolution mass spectrometry data were converted to mzXML format using ProteoWizard tool MSconvert (version 3.0.10051, Vanderbilt University, United States) (Chambers *et al.*, 2012). The converted files were uploaded to the GNPS molecular networking server and analysed by the GNPS platform (Wang *et al.,* 2016). A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). All tandem mass spectrometry (MS/MS) fragment ions within +/- 17 Da of the precursor m/z was deleted and the precursor ion mass tolerance was set to 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da. The data was then analysed with a cut-off cosine score of above 0.7 and with more than 5 matched peaks. Furthermore, edges (connections) between two nodes (molecules e.g., secondary metabolite) were kept in the network if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the size of a molecular family was restricted to 100, and so edges with the lowest scores were sequentially removed to ensure the molecular family size was below this threshold. The mass spectra of the molecules were then compared with GNPS' spectral libraries to identify their identity based on similar fragmentation patterns. The library spectra were filtered in the same manner as the input data. For matches to be retained they had to have a score above 0.7 and at least 5 matched peaks (Wang *et al.*, 2016)

2.14 Statistical analysis

Analysis of variance (ANOVA) is an extension of an unpaired t-test and compares differences between means of groups treated independently. A one-way ANOVA with multiple comparison Dunnett's post-test were performed on the mean of two independent biological experiments performed in triplicate. An unpaired t-test, which compares the means of two independent groups to determine if there is a significant difference between the two, was also conducted on selected data. IC_{50} values were determined by non-regression analysis of log inhibitor vs dose response. However, the log inhibitor dose response curve fit did not provide IC_{50} values for Antimycin and Valinomycin and hence a non-regression fourth order polynomial equation was used to identify the curve that best models the arrangement of data points. All analysis was performed using GraphPad Prism^{TM} 8.4.3. Statistical significance was indicated as *, **, *** and **** for p-values less than 0.05, 0.01, 0.001 and 0.0001, respectively.

Chapter 3: Identifying novel active compounds and secondary metabolites produced by strains of filamentous actinobacteria that impact HIV-1 infection

3.1 Introduction

Despite significant reduction in HIV incidence since the pandemic began, current ARV treatment is still not ideal (Nega *et al.*, 2020). Although viral load is suppressed with ARVs, long-term exposure is associated with serious non-AIDS events (SNEAs) including cardiovascular issues, renal disorders and osteoporosis (Chawla *et al.*, 2018), and despite ART, millions are still newly infected every year. In addition, no vaccine has yet been developed due to the high mutation rate of the virus and the extensive genetic diversity of core genes such as *env* (Rudometov *et al.*, 2019). Therefore, other approaches are needed to counter the continuing global crisis of HIV infection.

NPs, specifically bioactive secondary microbial metabolites, have been utilized for thousands of years in the effective treatment of infectious diseases. Filamentous actinobacteria and their ability to produce versatile bioactive secondary metabolites have been at the forefront of antibiotic and antifungal research, and more recently anti-viral research, especially in the context of HIV (Gordon *et al.*, 2020). Studies, such as those conducted by Chen *et al.* (2018) and Liu *et al.* (2013), suggest that microbial metabolites show promise as effective HIV-1 infection preventative measures.

Therefore, this study screened 23 indigenous strains of filamentous actinobacteria for non-cytotoxic active anti-viral compounds that inhibit HIV-1 infection. Secondary bioactive metabolites were extracted by LLE and tested for activity by determining their effect on PSV entry of a reporter cell line (TZM-bl cells). Bacterial extracts with anti-viral activity were further analyzed on a triple quadrupole time-of-flight instrument (QTOF) and GNPS to identify the secondary metabolites with potential anti-HIV infection activity.

3.2 Results

3.2.1 Dimethyl sulphoxide (DMSO) does not interfere with the pseudovirus infection assay

To extract bioactive metabolites from filamentous actinobacteria LLE was performed using ethyl acetate, hexane, and methanol.

Both DMSO and methanol significantly decreased PSV infection efficiency at a concentration of 0.1% *v/v* without affecting cell viability (Figure 3.1). To determine whether the observed reduction in infection efficiency was due to direct inhibition of viral infectivity or via inhibition of the luciferase assay, the assay was repeated in the presence of 0.05% DMSO and methanol and there was no significant decrease in the percentage infection in the presence of 0.05% *v/v* DMSO or methanol (Figure 3.1 A). This suggested that at this concentration, the solvents no longer affected the luciferase assay. Furthermore, both concentrations of solvents had no effect on cell viability (Figure 3.1 B). Compared to the cells-only control, PSV in the absence of solvent significantly decreased cell viability possibly due to virus-induced cell death. However, the decrease was not observed in the presence of solvents suggesting that at low levels they might have enhanced cell proliferation as indicated for DMSO (Singh,M *et al.,* 2017), camouflaging the cytotoxic effects of PSV infection. As methanol evaporates more rapidly than DMSO, DMSO was selected as the resuspension solvent and all samples were diluted to a maximum concentration of 0.05% *v/v* DMSO.

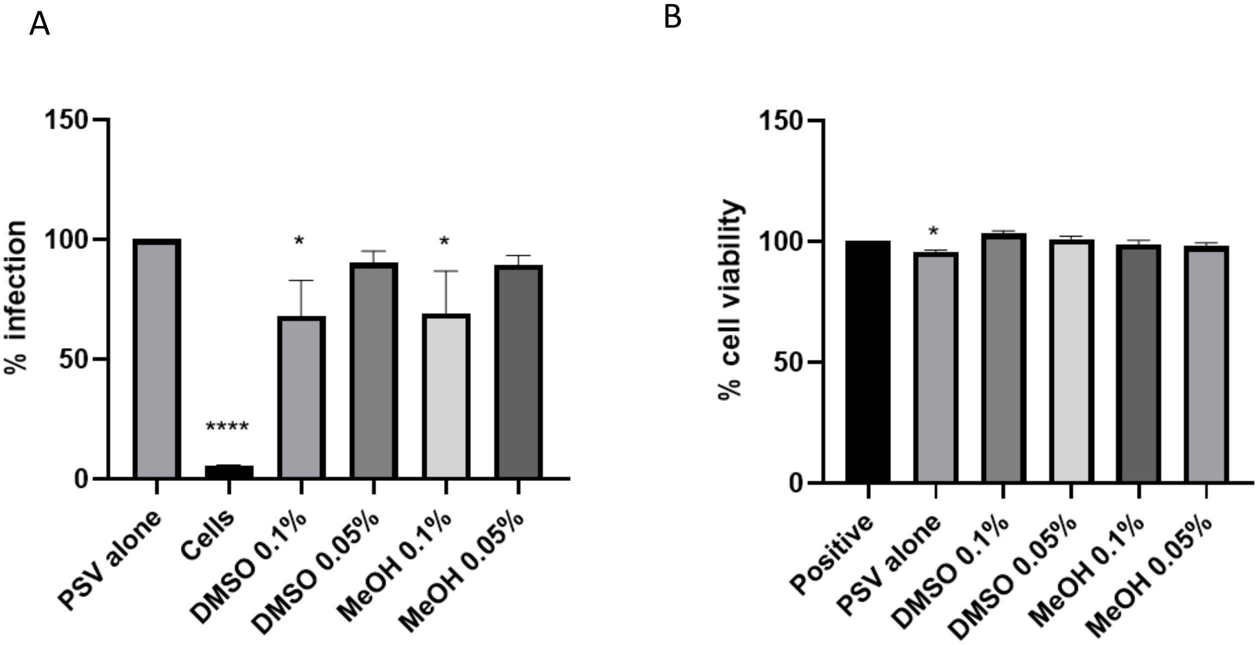

Figure 3.1: **The effect of DMSO and Methanol on PSV entry and cell viability. (A)** TZM-bl cells (5x10^4 cells/mL) were infected with 200 TCID$_{50}$ PSV in the presence of DMSO or methanol at 0.1% *v/v* or 0.05% *v/v* and changes in entry efficiency were determined by relative light units (RLU). To compare data across independent experiments, infection was calculated relative to the positive control (PSV alone). The cells only (negative) control comprising only TZM-bl cells and growth medium are also shown. **(B)** An MTT assay was performed to determine the effects of the solvents on TZM-bl cell viability. The percentage viability was calculated relative to the cells only control (Positive). PSV alone represents TZM-bl cells with PSV only in the absence of solvents. Bars represent the mean of two independent experiments with error bars indicating standard deviation. Statistical analysis was carried out using GraphPad one-way ANOVA and post-hoc Dunnett's multiple comparison test. *, **, *** and **** represent P<0.05, 0.01, 0.001 and 0.0001 respectively.

3.2.2. Screening of the filamentous actinobacteria strains

To determine whether filamentous actinobacteria produced bioactive metabolites with anti-viral activity, PSV infection was carried out in the absence and presence of 50 µg/mL and 25 µg/mL of each actinobacteria crude extract. Table 3.1 shows the actinobacteria strains tested, inhibition of PSV infection at 25 µg/mL crude extract, cell viability and the compound identified from the GNPs database.

Table 3.1: Identification of compounds potentially associated with actinobacteria strains cytotoxicity and ability to inhibit PSV infection

Strain	Media [DSMZ (D) or Bennett's (B)]	Inhibition (25 µg/mL)	Cytotoxicity	Identified compound	Cosine score	Mass Difference (Da)
Streptomyces strain B1	B	No	No	Oleandomycin Valinomycin	0.84 0.75	2.279 0.008
Streptomyces strain B2	B	Yes (**)	Yes (***)	Pyrromycin	0.73	15.998
Nocardia strain B10S Fri	B	Yes (*)	No	Antimycin A3	0.94	14.016
Streptomyces strain #15	B	Yes	No	Anisomycin	0.72	0.045
Micromonospora strain M8	D	Yes (***)	No	Monactin	0.84	0.215
Streptomyces strain SK5	B	No	Yes (**)	Dehydroxynocardamine	0.78	0.269
Streptomyces strain PR3	D	Yes (*)	Yes (***)	Monactin Adenine Tunicamycin B Valinomycin	0.82 0.87 0.91 0.82	0.000 0.001 14.015 28.029
Streptomyces strain PR10	D	ND	Yes (***)	Actinomycin D	0.80	1.232
Streptomyces strain CW2	D	ND	Yes (****)	Valinomycin Oleandomycin	0.75 0.84	0.008 2.279
Streptomyces strain CW5	D	ND	Yes (****)	Streptomycin	0.93	17.995
Micromonospora strain M27	D	No	Yes (*)	ND	N/A	N/A
Streptomyces strain C3	D	No	Yes (***)	ND	N/A	N/A
Streptomyces strain C23	D	No	No	ND	N/A	N/A

Streptomyces strain 1J1	D	No	No	ND	N/A	N/A
Streptomyces strain Y10	B	ND	ND	ND	N/A	N/A
Streptomyces strain Y12	B	No	Yes (***)	ND	N/A	N/A
Streptomyces strain Y30	B	ND	Yes (****)	ND	N/A	N/A
Streptomyces strain MV32	D	ND	Yes (****)	ND	N/A	N/A
Nonomurea candida QMC26	D	No	Yes (*)	ND	N/A	N/A
Microbispora SMA HA3	D	No	No	ND	N/A	N/A
Streptomyces strain PR4	B	ND	Yes (****)	ND	N/A	N/A
Micromonospora tulbaghiae	D	No	Yes (****)	ND	N/A	N/A
Streptomyces strain JBGM7	B	Yes (**)	Yes (***)	ND	N/A	N/A

**ND stands for not determined*

, **, * and **** represent P<0.05, 0.01, 0.001 and 0.0001 respectively.*

Many compounds were identified however majority were ruled out as they were due to components of the media or were known to not be associated with this investigation. Those shown are the ones with potential to contribute to the inhibition seen.

3.2.2.1 Impact of crude extracts on pseudovirus entry and cell viability

The impact of the 23 strains on PSV infection was highly variable, with the extracts of 17 strains significantly inhibiting PSV infection, (range: 0 to 46%), at a concentration of 50 µg/mL and the extracts of 18 strains significantly reducing cell viability. The extracts of 7 strains did not significantly decrease PSV infection at 50 µg/mL and 3 of these did not significantly reduce cell viability (Figure 3.2 A and C). *Streptomyces* strains, PR3, MV32 and JBGM7 showed significant inhibition of PSV infection at 50 µg/mL and 25 µg/mL (Figure 3.2 A and B), and significantly reduced cell viability at both concentrations, suggesting that these strains are producing cytotoxic compounds (Figure 3.2 C and D) and are not ideal candidates for HIV inhibition. However, this does not exclude the possibility that these strains may produce secondary metabolites with anti-viral activity in addition to other cytotoxic compounds.

Interestingly, *Streptomyces* strain Y12, *Streptomyces* strain C3, *Kribella speibonae* SK5 and *Micromonospora* strain M27 did not show PSV inhibition at 25 µg/mL (Figure 3.2 B), butdid show a significant impact on cell viability at this concentration (Figure 3.2 D). This suggests that despite these strains producing cytotoxic compounds, PSV infection was not affected. It is possible that the potency of the cytotoxic compounds was not high enough to reduce the number of viable cells needed to sustain PSV infection or the extract affected the MTT assay itself.

Only the extracts of 3 strains significantly inhibited PSV infection without a significant impact on cell viability. These strains, *Nocardia* strain B10 Fri, *Streptomyces* strain #15 and *Micromonospora* strain M8 showed significant inhibition of PSV entry at 50 µg/mL and 25 ug/mL, although inhibition by *Streptomyces* strain #15 did not reach significance at the lower concentration (Figure 3.2 A and B). The extracts from these strains did not result in significant reduction in cell viability (Figures 3.2 C and D), suggesting that these strains could be producing non-cytotoxic, active anti-viral metabolites. The media used to culture the strains (either DSMZ or Bennett's) were analyzed on the GNPS database to identify whether the compounds from the strains could have been due to their presence in the media. We found that none of the products displaying activity were identified in either medium.

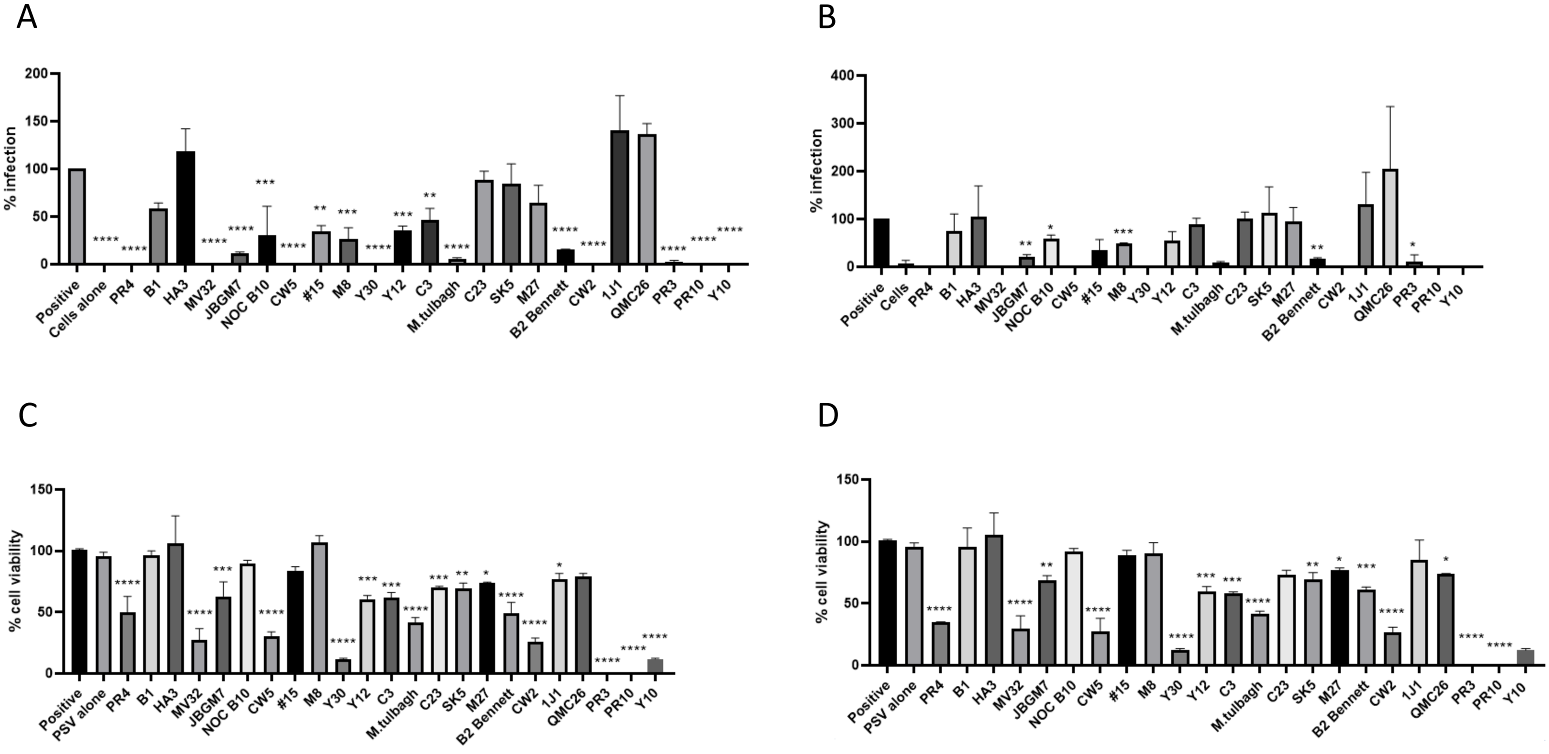

Figure 3.2: **The effect of actinobacteria extracts on PSV entry and cell viability.** Twenty-three strains of filamentous actinobacteria were cultured in either GPHF-Medium (DSMZ) or 174 Bennett's broth for 10 days at 30°C and shaking at 100 rpm before methanol and ethyl acetate were added to the abiotic culture medium. After separation, the ethyl acetate layer was removed and left to dry and resuspended in DMSO for testing. TZM-bl cells ($5x10^4$ cells/mL) were infected with PSV equivalent to 200 TCID$_{50}$ in the presence of **(A)** 50 µg/mL and **(B)** 25 µg/mL of the extract from each strain and incubated for 48hrs at 37°C, 5% CO_2. Percentage infection (calculated by comparing the relative light units (RLU) of each infection relative to the RLU of PSV only control). The negative control (cells alone) containing only cells and growth medium is also shown. TZM-bl cells ($5x10^4$ cells/mL) were infected with PSV equivalent to 200 TCID50 in the presence of **(C)** 50 µg/mL and **(D)** 25 µg/mL of the extract from each strain and incubated for 48hrs at 37°C, 5% CO_2. An MTT assay was performed to determine the effects of the bacterial extracts on TZM-bl cell viability. The percentage cell viability was calculated by dividing the fold-change in absorbance of each treatment to the fold change in absorbance of the cells only control (positive). Each biological repeat was carried out in triplicate. Bars represent the mean of two independent experiments with error bars indicating standard deviation. Statistical analysis was carried out using GraphPad one-way ANOVA and post-hoc Dunnett's multiple comparison test, as well as an unpaired t-test. *, **, *** and **** represent $P<0.05$, 0.01, 0.001 and 0.0001 respectively.

3.2.3. The identification of active anti-viral metabolites

Of the 23 strains, 10 strains were selected according to varying characteristics: 1) non-cytotoxic strains showing significant anti-viral activity (*Nocardia* strain B10 Fri, *Streptomyces* strain #15 and *Micromonospora* strain M8), 2) cytotoxic strains with no anti-viral activity (B1, CW5, CW2, PR10) and 3) cytotoxic strains with anti-viral activity (PR3, B2, SK5) (Table 1) The extracts were analyzed by high resolution mass spectrometry using a HPLC- QTOF to identify potential NPs with anti-HIV activity. Thereafter, the mass spectral data was compared to the GNPS database for the identification and confirmation of known bioactive NPs (Table 1). The cosine score is an algorithmic quantification of the alignment of the product ion spectra of a known compound in the GNPS library and an unknown match (Wang *et al.,* 2016). The higher the cosine score, the more closely the two spectra match and the more structurally similar the unknown is to the library compound. A score close to 1 indicates an almost 100% match. The threshold used in this study was a score above 0.7. The mass difference is a quantification of the shift in spectral peaks when comparing the unknown spectra and the library spectra. The smaller the mass difference, the more likely the masses are aligned. Mass differences of specific values may be due to an adduct gained during ionization and before analysis by mass spectrometry or the addition of a methyl or phosphate group. Results from GNPS can compare and visualize the product ion spectra obtained from the query relative to the library.

GNPS analysis identified 11 known compounds, including Dehydroxynocardamine, Adenine and 9 antimicrobials, with Valinomycin being the most common. The mass differences were low indicating an accurate match for all hits except Pyrromycin in *Streptomyces* strain B2, Antimycin in *Nocardia* strain B10S Fri, Tunicamycin and Valinomycin in *Streptomyces* strain PR3, and Streptomycin in *Streptomyces* strain CW5 (Table 1). The mass difference between the sample and library query for Streptomycin from strain CW5 is likely due to a difference in adducts, with the mass difference of 17.99 Da representing an ammonium (NH_4^+) ion. Ammonia is present in the aqueous mobile phase and ammonium adducts are often detected. A mass difference of 28 Da in Valinomycin in strain PR3 represents an analogue with the loss of 2 CH_2 groups with a mass of 1100 Da and has been identified in previous studies (Watson *et al.*, 2021).

The partial match to Tunicamycin B is likely a match to Tunicamycin A, its analogue, with a mass difference of 14 Da (Takatsuki *et al.*, 1977). The Antimycin A3 partial match with a 14 Da mass difference is likely Antimycin A11 (Hosotani *et al.*, 2005). The partial match to Pyrromycin is likely its analogue 1-deoxypyrromycin which has an oxygen group missing accounting for the mass difference of 16 Da (Oki *et al.*, 1979). The low mass differences between Oleandomycin in *Streptomyces* strain B1 and Actinomycin in *Streptomyces* strain PR10 maybe due to mass errors from the different instruments used in this experiment and for the library spectra.

Figure 3.3 indicates how Valinomycin was identified by GNPS. The black peaks show the mass peaks of the product ions of the unknown product (query) and the green peaks show the mass peaks of product ions of known antimicrobial Valinomycin (library). The alignment of these peaks indicates that the product seen in the bacterial sample is in fact Valinomycin because it aligns completely to the product ions of Valinomycin.

As far as we are aware, Dehydroxynocardamine does not have any known antimicrobial activity. It was suggested that GV produces Adenine that inhibits HIV infection (Taneva *et al.*, 2018) but Klatt *et al.* (2017) reported that Adenine was produced as a byproduct of tenofovir metabolism (Klatt *et al.*, 2017) and was thus only produced under certain circumstances. Therefore, we did not include Dehydroxynocardamine and Adenine in this study and focused on the antimicrobials. Firstly, antimicrobials were identified that were unique to strains that were non-cytotoxic and had anti-viral activity but were not produced by strains that were both cytotoxic and inhibitory. However, there was no one compound common to all non-cytotoxic, bioactive strains and Anisomycin and Antimycin were the only antimicrobials unique to *Streptomyces* strain #15 and *Nocardia* strain B10S Fri, respectively. As we cannot exclude the possibility that strains producing anti-viral antimicrobials are also producing other cytotoxic compounds, we selected a range of antimicrobials produced by strains with varying cytotoxicity and inhibitory activity.

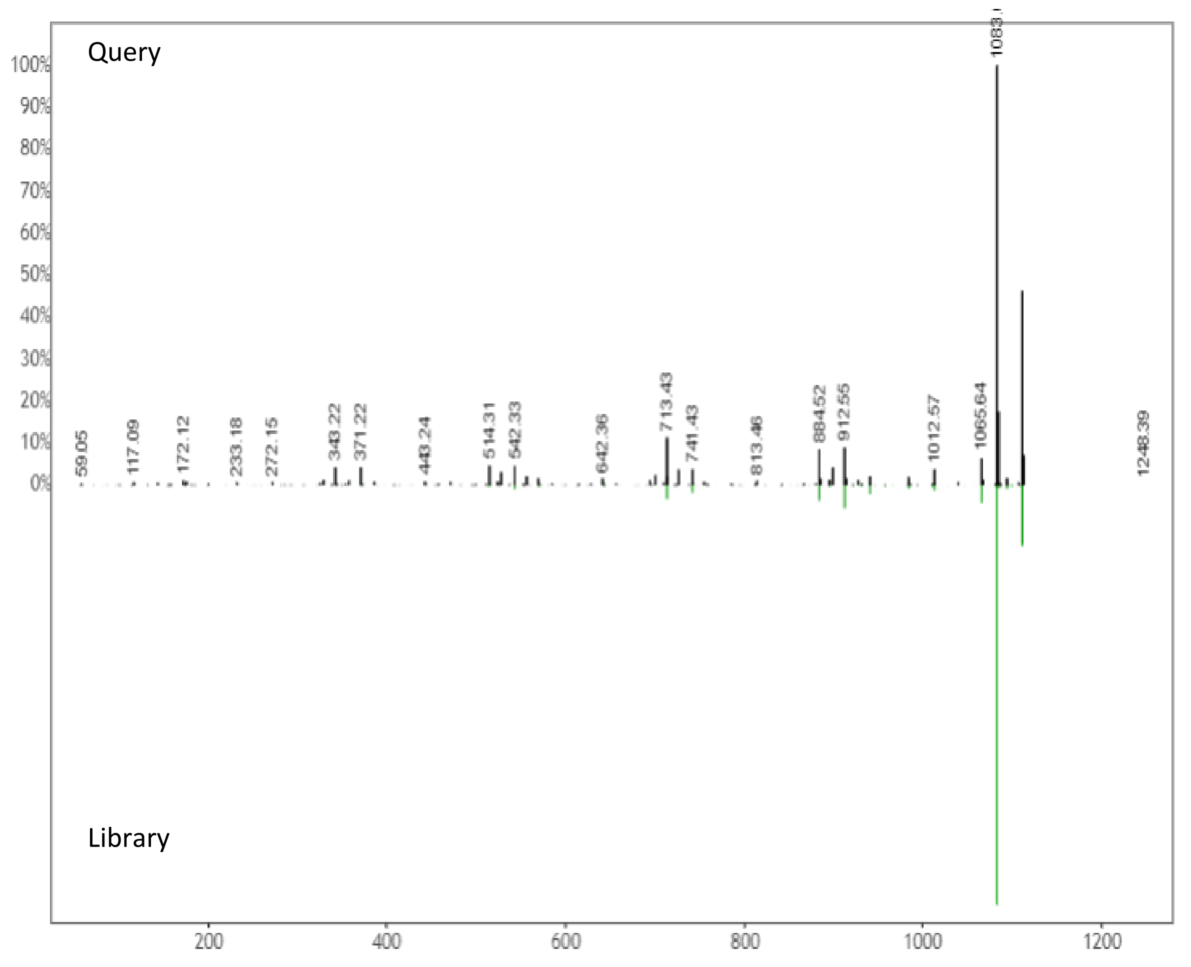

Figure 3.3: Alignment of product ion spectra of an unknown compound (query) from *Streptomyces* strain B1 grown in Bennett's and that of the known library compound Valinomycin (library).

3.2.5. Impact of antimicrobials on PSV infection

The antimicrobials identified from the GNPS database indicated that these NPs could possibly be the compounds inhibiting PSV infection (Figure 3.2). The aim of this section was to identify the range of antimicrobial concentration that inhibited PSV infection without affecting cell viability. A NP with high cytotoxic activity is unlikely to be a good candidate for inhibition of HIV infection *in vivo*.

3.2.5.1 Antimicrobials inhibit PSV infection

Initially 4 antimicrobials were tested, Actinomycin D, Valinomycin, Antimycin and Puromycin from 1 µg/mL to 50 µg/mL and all 4 inhibited PSV entry (Figure 3.4). As Actinomycin D binds both double and single stranded DNA (Jeeninga *et al.*, 1998) it inhibits HIV-1 strand transfer during reverse transcription with an IC_{50} of approximately 0.3 µM (Guo *et al.*, 1998; Jeeninga *et al.*, 1998). We found that Actinomycin D significantly inhibited PSV entry at 0.008 µg/mL (0.006 nM) and 0.0016 µg/mL (0.0012 nM) (Figure 3.5 A) which was much lower than the IC_{50} reported before.

As reviewed by Huang *et al.* (2021), Valinomycin has multiple bioactivities including antifungal, antibacterial and anti-tumor activities, and most importantly for our context, anti-viral activity with IC_{50} values that ranged from 1.5 nM to 971 nM depending on the virus (Huang *et al.*, 2021). However, due to variation between experiments, we could not identify the most effective range of concentrations to determine the IC_{50} of Valinomycin although there was a tendency for inhibition to decrease with decreasing antimicrobial concentration (Figure 3.5 B).

As there was apparent inhibition without cytotoxicity at low concentrations, we determined the IC_{50} for 5 antimicrobials: Valinomycin, Puromycin, Actinomycin D, Antimycin and Anisomycin.

3.2.5.2 Cytotoxicity of antimicrobials

Although Valinomycin, Puromycin, Actinomycin D, and Antimycin inhibited PSV entry (Figure 3.4), we needed to confirm that the apparent inhibition was not due to cytopathic effects. Except for Antimycin at 1 µg/mL, all 4 antimicrobials significantly impacted cell viability (Figure 3.4 B). Interestingly, Antimycin at 1 µg/mL showed significant (p-value < 0.01) inhibition of PSV entry with no significant impact on cell viability, suggesting that concentrations at 1 µg/mL and lower, might not be cytotoxic to TZM-bl cells but could still have anti-HIV activity. Shibata (1997) reported that Antimycin A was cytotoxic to numerous cell lines with an IC_{50} range between 0.015 µg/mL (27nM) and 0.063 µg/mL (114 nM) (Shibata,1997), suggesting that concentrations lower than 0.015 µg/mL might have no impact on the cell viability of TZM-bl cells.

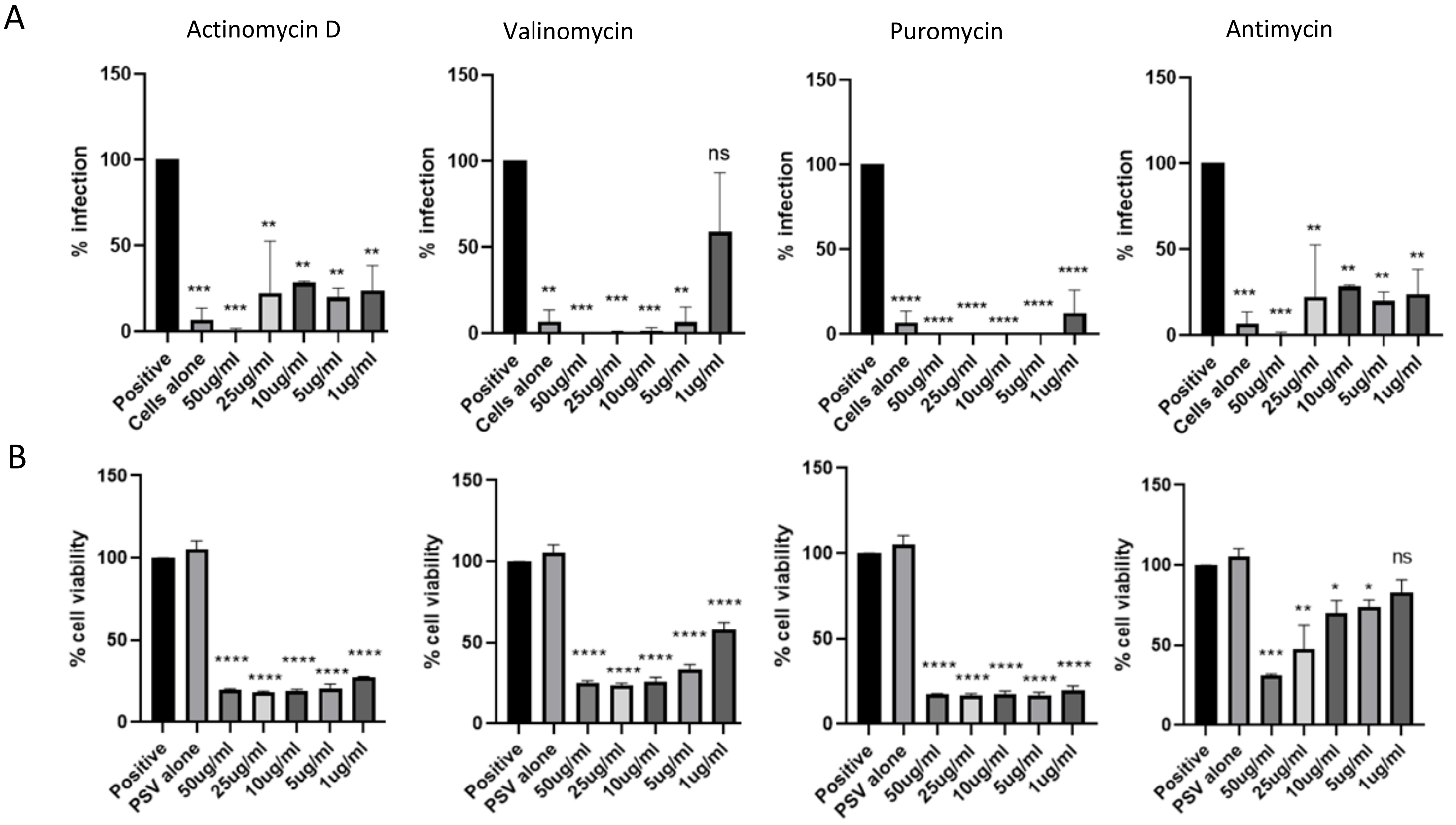

Figure 3.4: **The effect of antimicrobials on pseudovirus entry and cell viability.** Four antimicrobials were diluted to 50 µg/mL, 25 µg/mL, 10 µg/mL, 5 µg/mL and 1 µg/mL (**A)** Percentage infection (calculated by comparing the relative light units (RLU) of each infection relative to the RLU of PSV only control (positive). The negative control (cells only) is also shown. TZM-bl cells ($5x10^4$ cells/mL) were infected with PSV equivalent to 200 $TCID_{50}$ in the presence of **(B)** 50 µg/mL, 25 µg/mL, 10 µg/mL, 5 µg/mL and 1 µg/mL of each antibiotic and incubated for 48hrs at 37°C, 5% CO_2. The percentage cell viability was calculated by dividing the fold-change in absorbance of each treatment to the fold change in absorbance of the cells only control (positive). Bars represent the mean of two independent experiments with error bars indicating standard deviation. Statistical analysis was carried out using GraphPad one-way ANOVA and post-hoc Dunnett's multiple comparison test. *, **, *** and **** represent P<0.05, 0.01, 0.001 and 0.0001 respectively.

Both Puromycin and Actinomycin D inhibit protein synthesis and are highly cytopathic with CC_{50} (cytotoxicity concentration at 50% of maximum) of 6.2 µM and 1300 µM in HepG2 cells, respectively whereas in Hela cells, Puromycin was cytotoxic at 0.9 µM (Chan *et al.*, 2017). We determined that Puromycin up to 1 µg/mL (2 µM) significantly reduced TZM-bl viability, suggesting that much lower concentrations be used when determining inhibition of PSV infection.

Previously, it was shown that Actinomycin D reduced A10 and HepG2 cell viability with an IC_{50} of 0.4 nM (Wu *et al.*, 2005) and 7.4 nM, respectively (Chan *et al.*, 2017). The approximately 20-fold difference in IC_{50} suggested that it affected cell viability differently according to cell line. Wild-type Hela cells were shown to be highly susceptible to Actinomycin D with cell death dependent on dose and time of exposure within the range 0.01-2.5 µg/mL, after which maximum death occurred (Sawicki and Godman *et al.,* 1971). We found that Actinomycin D did not significantly reduce TZM-bl cell viability at 0.008 µg/mL (6.4 nM) (Figure 3.5 C) potentially because of cellular modifications of TZM-bl cells as the authors report that Hela S3 cells were slightly more resistant to Actinomycin D than wild-type.

Similarly, Valinomycin also had varying cytotoxic effects on different cancer cell lines: A2780, MCF-7, HepG2 and C6 with IC_{50} values of 2.18, 1.77, 0.0008 and 0.0004 µM, respectively (Iacobazzi *et al.*, 2013) . Valinomycin was thus more cytotoxic than Actinomycin D in HepG2 cells. Despite inter-experimental variation, Valinomycin seemed to have no effect on TZM-bl cell viability between 0.008 µg/mL and 0.00032 µg/mL (Figure 3.5 D), a range (0.29 -7.2 nM) which included the IC_{50} values previously identified for other cancer cells.

Overall, according to these findings, Actinomycin D and Valinomycin are not cytotoxic to TZM-bl cells at 0.008 µg/mL whereas Antimycin did not significantly reduce cell viability at 1 µg/mL. We hypothesized that these values, within the context of IC_{50} values previously published, could guide the selection of the concentration range needed to determine the IC_{50} of the antimicrobials.

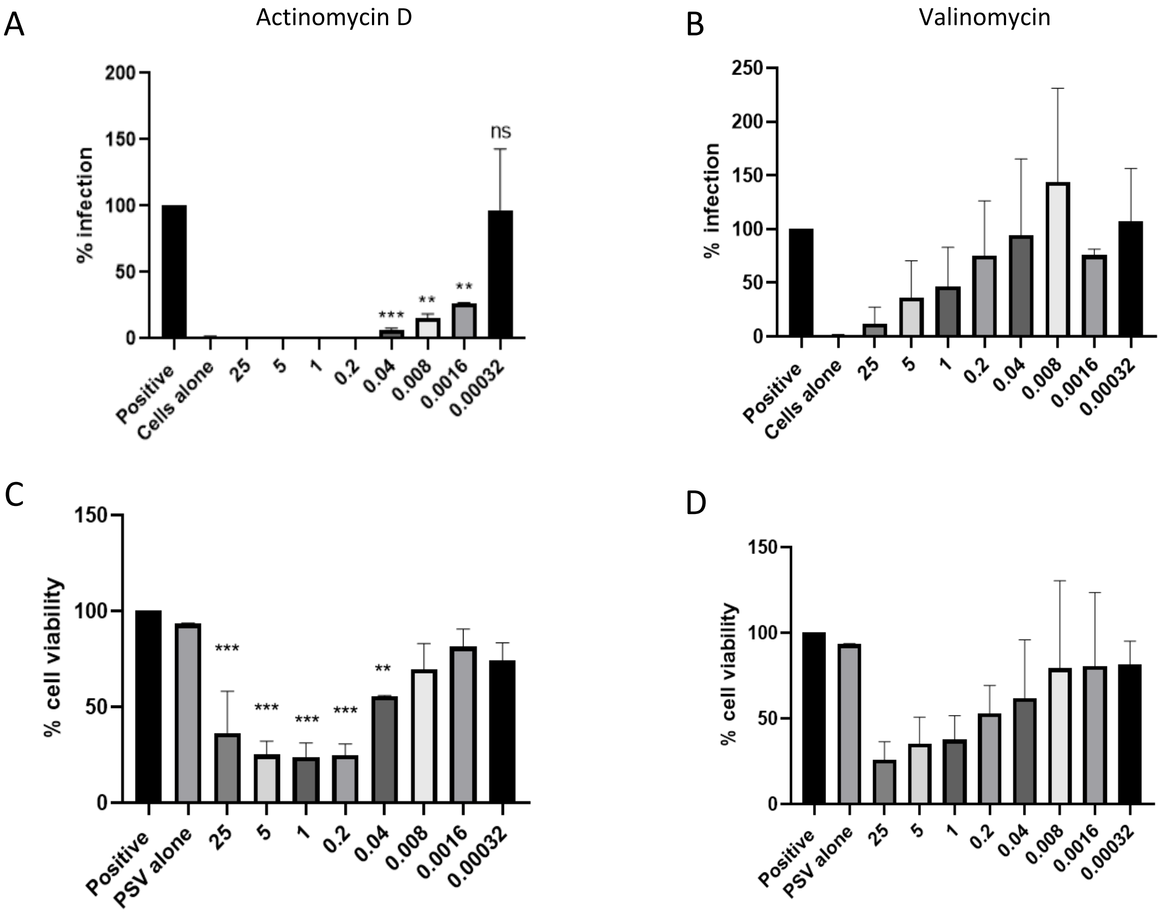

Figure 3.5: **The effect of antimicrobials on PSV entry and cell viability.** Two antimicrobials were diluted to 25 µg/mL, 5 µg/mL, 1 µg/mL, 0.2 µg/mL and 0.4 µg/mL, 0.008 µg/mL, 0.0016 µg/mL, and 0.00032 µg/mL. TZM-bl cells ($5x10^4$ cells/mL) were infected with PSV equivalent to 200 $TCID_{50}$ in the presence of these concentrations and were incubated for 48hrs at 37°C, 5% CO_2. (**A and B)** Percentage infection (calculated by comparing the relative light units (RLU) of each infection relative to the RLU of PSV only control (positive). The negative control (cells alone) is also shown. TZM-bl cells ($5x10^4$ cells/mL) were infected with PSV equivalent to 200 $TCID_{50}$ in the presence of **(C and D)** 25 µg/mL, 5 µg/mL, 1 µg/mL, 0.2 µg/mL and 0.4 µg/mL, 0.008 µg/mL, 0.0016 µg/mL, and 0.00032 µg/mL of each antibiotic and were incubated for 48hrs at 37°C, 5% CO_2. The percentage cell viability was calculated by dividing the fold-change in absorbance of each treatment to the fold change in absorbance of the cells only control (positive).Bars represent the mean of two independent experiments with error bars indicating standard deviation. Statistical analysis was carried out using GraphPad one-way ANOVA and post-hoc Dunnett's multiple comparison test. *, **, *** and **** represent P<0.05, 0.01, 0.001 and 0.0001 respectively.

3.2.6 IC_{50} of antimicrobials

The antimicrobials identified as being produced by the filamentous actinobacteria could possibly be used as anti-HIV treatments. To establish the IC_{50} of antimicrobials required to inhibit PSV infection, the antimicrobialswere tested at a concentration range from 1 µg/mL to 13.3 pg/mL (Antimycin and Anisomycin); 0.04 µg/mL to 13.3 pg/mL (Puromycin and Valinomycin) and 0.008 µg/mL to 13.3 pg/mL (Actinomycin D) based on the range of concentration cytotoxic to TZM-bl cells for each of the antimicrobial (Figure 3.4 and 3.5). These concentrations correspond to Actinomycin D at a range between 6.4 nM to 0.012 nM, Valinomycin at 36 nM to 0.012 nM, Antimycin at 1823 nM to 0.024 nM, Puromycin at 73 nM to 0.024 nM and Anisomycin at 3769 nM to 0.05 nM.

Table 3.2:

Comparison between the IC_{50}s of commercial antibiotics identified by screening filamentous actinobacteria and published CC_{50} and IC_{50}s values

Antimicrobial	**IC_{50} below cytotoxicity**	**IC_{50} full range**	**CC_{50} published**	**IC_{50} published**
Actinomycin D	27.8 nM	9.15 nM	1300 µM (Chan *et al.*, 2017)	#0.3 µM (Guo *et al.*, 1998; Jeeninga *et al.*, 1998)
Valinomycin	*N/A*	*N/A*	2.18 to 0.0008 µM (Iacobazzi *et al.*, 2013)	1.5 to 971 nM (Huang *et al.*, 2021)
Anisomycin	*ND*	61.6 nM	*75 nM (Lee *et al.*, 2005)	32.2 to 7.9 nM (Quintana *et al.*, 2020)
Puromycin	0.53 nM	67.7 nM	6.2 to *0.9 µM (Chan *et al.*, 2017)	N/A
Antimycin	*NA*	*NA*	27 to 114 nM (Shibata,1997)	3 nM (Raveh *et al.*, 2013)

**Determined in Hela cells*
 # HIV infection assay
ND: not determined,
NA: not applicable (Statistical analysis did not provide an IC_{50} *value)*

Actinomycin D inhibited PSV infection with an IC_{50} value of 27.8 nM (Table 2) which was much lower than previously reported for HIV infection (Guo *et al.*, 1998; Jeeninga *et al.*, 1998) (Table 2). When PSV inhibition was plotted against the whole range of antimicrobial concentrations (Figure 3.6) it was likely that there was no inhibition at low concentration and the IC_{50} of 9.15 nM was due to cell death.

The non-cytotoxic range of Puromycin generated an IC_{50} of 0.53 nM and when PSV inhibition was plotted across concentrations that included those identified as cytotoxic (13.3 pg/mL – 80 µg/mL), the IC_{50} increased to 67.7 nM. Furthermore, Puromycin showed a rapid increase in inhibition (0 to 99%) within a 1.5-fold change in concentration (0.04 µg/mL to 0.06 µg/mL) (Figure 3.6). This could suggest a binary dose response curve where low concentrations have no effect and once a threshold is reached, all higher concentrations have the same effect (Vandenberg *et al.*, 2012). Previously, the cytotoxicity IC_{50} of Puromycin in Hela cells was reported as 0.9 µM (Chan *et al.*, 2017), more than 10-fold higher than the PSV inhibition IC_{50} reported here, suggesting that the inhibition observed from 0.06 µg/mL (0.13 µM) to 80 µg/mL (72 µM) (Figure 3.6) was due to cell death and skewed the IC_{50} value. However, it is also possible that assay differences between studies influenced the analysis.

The inhibition of PSV infection by Antimycin was highly variable (6.1-73,5%) and did not correlate with increasing antimicrobial concentration (Figure 3.6). This result was surprising as we initially observed significant inhibition at 1 µg/mL without affecting cell viability (Figure 3.4) whereas here, Antimycin only inhibited PSV infection by 7.9% at this concentration. The variation between experiments is likely due to experimental error although the apparent increase in inhibition at high concentrations [84% at 50 µg/mL (45 µM) and 99% at 80 µg/mL (72 µM)] is likely due to cytotoxicity (Figure 3.6). As the dose response curve for all concentrations of Antimycin reported "ambiguous" IC_{50} values, we carried out fourth order polynomial analysis to identify a curve that best modeled the spread of data (r^2= 0.8591) and identified a non-monotonic distribution which has been shown for a NNRTI HIV inhibitor (Zhang *et al.*, 2013). Similarly, Valinomycin showed highly variable inhibition especially at lower concentrations, although the observed inhibition seen at the higher concentrations (45 µM and 72 µM) is likely due to cell death (Figure 3.6) as previously it was shown that 10 µM Valinomycin rapidly caused apoptosis in Hela cells after 6 hrs

(Zhang *et al.*, 2014). PSV inhibition also followed a polynomial distribution (r^2= 0.7823), and we were thus not able to determine an IC_{50} for Antimycin and Valinomycin (Table 2).

Anisomycin dose response curve yielded an IC_{50} of 61.6 nM. Anisomycin has been showed to display anti-viral activity against Dengue and Zika viruses by inhibiting eukaryotic protein synthesis, thereby preventing viral protein expression (Quintana *et al.*, 2020). Virus inhibition IC_{50} values ranged from 32.2 nM to 7.9 nM in 3 different human cell lines, whereas the value we established was much higher (Table 2). Anisomycin cytotoxicity in Hela cells has been reported as an IC_{50} of 75 nM which is very similar to the IC_{50} for PSV inhibition determined in this study (Lee *et al.*, 2005), suggesting that Anisomycin might not be inhibiting PSV infection but reducing TZM-bl cell viability. However, as we have no data on the effect of Anisomycin on cell viability, additional analysis is required.

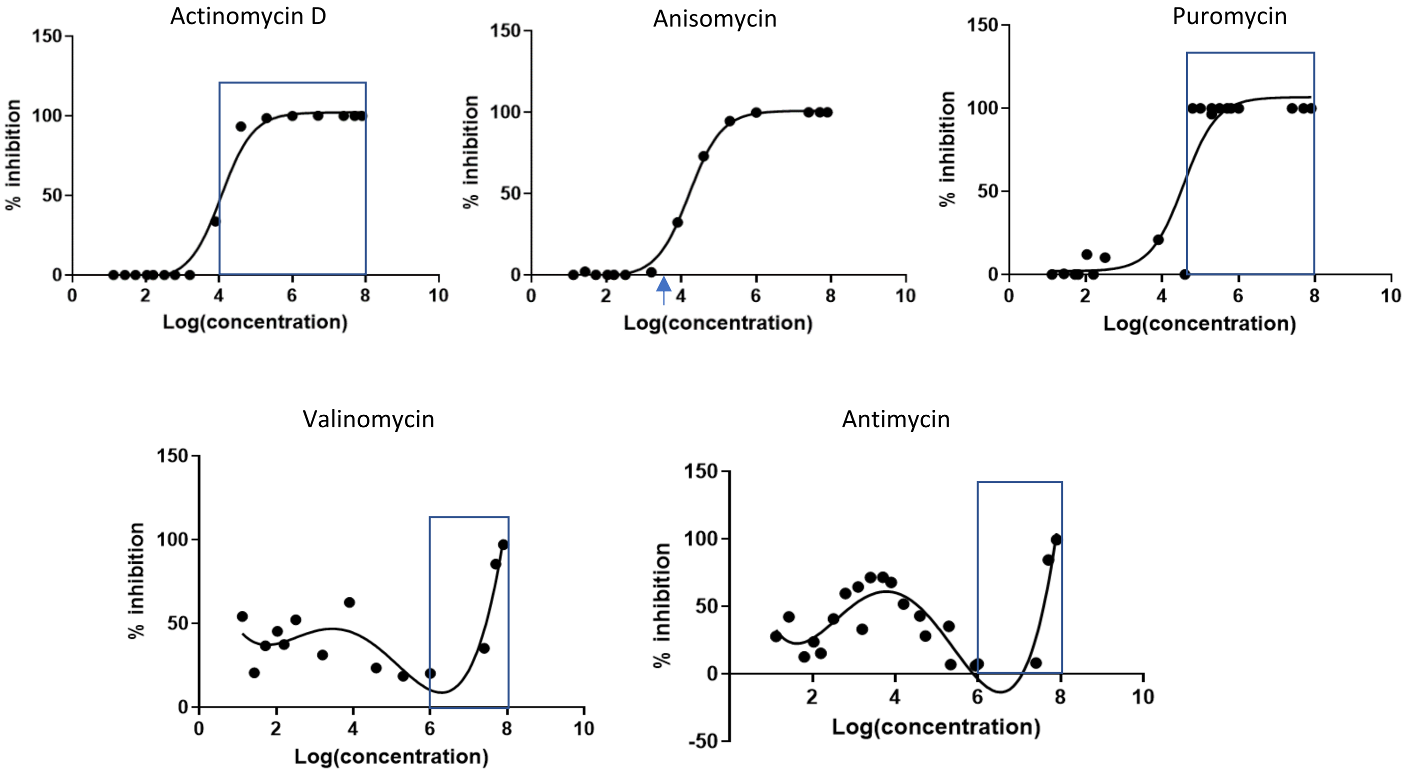

Figure 3.6: **Establishing IC_{50} for antimicrobials on HIV infection.** Five antimicrobials were tested from 80 µg/mL to 13.3 pg/mL. TZM-bl cells ($5x10^4$ cells/mL) were infected with PSV equivalent to 200 $TCID_{50}$ in the presence of Actinomycin D, Valinomycin, Puromycin, Antimycin and Anisomycin and were incubated for 48hrs at 37°C, 5% CO_2. Percentage inhibition (calculated by comparing the relative light units (RLU) of each infection relative to the RLU of PSV alone and subtracting from the PSV alone) is shown. The concentrations shown to reduce cell viability is indicated by a blue box. The Anisomycin IC_{50} (75 nM) known to cause cytotoxicity in Hela cells is shown by a blue arrow (Lee *et al.,* 2005). Statistical analysis was carried out using GraphPad non-linear regression of log(inhibitor) vs dose response. For Antimycin and Valinomycin non-regression polynomial curve fit analysis was carried out.

3.3 Discussion

HIV infection remains one of the leading causes of death in Sub-Saharan Africa despite the development of ARVs (UNAIDS 2020). Stigma, poor adherence, and debilitating side effects can lead to increased drug resistance especially in low economic countries where access to drugs impacts the effectiveness of ARVs (Van Der Straten *et al.*, 2012; Chawla *et al.*, 2018; Ford *et al.*, 2020). For example, the Qwa Qwa region in South Africa experienced an unreliable and interrupted supply of ARVs, impacting virus reactivation and patient mortality (Mokheseng *et al.*, 2017). This highlights the need to identify novel compounds with fewer side effects and lower cost of production. It is possible that NPs from indigenous sources might be more cost effective than current ARVs although this depends on the nature and availability of the NP. The aim of this project was to determine whether filamentous actinobacteria strains produce secondary metabolites that inhibit HIV infection. Thus far, no bacterial secondary metabolites have been identified that inhibit HIV entry.

The confirmation of novel NPs as antimicrobial agents requires identifying the therapeutic window where the compound is an effective inhibitor without causing harm to the host (Tamargo *et al.,* 2015). Therefore, the initial objective was to identify bacterial strains that produced NPs that inhibited PSV infection without cytotoxicity. LLE was used to extract bioactive metabolites from Actinobacteria. The separation of metabolites is based on their relative solubilities in two different immiscible liquids and once separated, the samples were dried down and resuspended in 0.1 % DMSO and methanol for further testing. DMSO and methanol are solvents routinely used in *in vitro* cell-based assays (Timm *et al.*, 2013). There was a decrease in infectivity but not cell viability, suggesting that the solvents were either affecting the ability of the virus to infect TZM-bl cells and/or it was affecting the luciferase reporter assay. As it has been shown that DMSO can have a positive effect on HIV infection (Seki et al., 1996), it is likely that DMSO is affecting the luciferase assay as previously described (Connelly et al., 2012). To ensure that the resuspension solvent did not affect the PSV infection assay and/or cell viability, 0.05% and 0.1% DMSO and methanol were added to the infection assay and changes in luminescence were measured. We found that DMSO was the most suitable solvent to use as it was not cytotoxic at low concentrations and did not evaporate.

The extracts of 3 out of 23 strains (*Nocardia* strain B10 Fri, *Streptomyces* strain #15 and *Micromonospora* strain M8) inhibited HIV infection without significantly affecting cell viability. As expected, GNP analysis identified a number of small molecules that could have bioactivity including Antimycin, Anisomycin and Monactin which are antimicrobials used in the treatment of various diseases and clinical research. The entry of Ebola virus, SARS-CoV and MERS-CoV transcription was inhibited by teicoplanin and glycopeptide antibiotics, including dalbavancin, oritavancin and telavancin (Zhou et al. 2016). As it has been shown that commercial antibiotics could also have antiviral activity, we decided to focus on them. Monactin, the parent compound of a group of ionophore antibiotics known as macrotetrolides (Woo *et al.,* 1999), was also produced by another strain, PR3, which was cytotoxic to TZM-bl cells and which also produced Valinomycin, Tunicamycin and Adenine. As Monactin was not unique to a strain that inhibited PSV infection without reducing cell viability and as it was not available, it was not analyzed in this study.

Anisomycin binds to the 60s ribosomal subunit, inhibiting peptide bond formation and subsequently protein synthesis (Y. Li *et al.*, 2018). It also inhibits cell proliferation by promoting the expression of p53, p21 and p27 in Jurkat T cells (Yu *et al.*, 2013). Antimycin is an inhibitor of the mitochondrial respiratory chain (Li-shar *et al.*, 2005). These mechanisms of action suggest that these antimicrobials only target eukaryotic cellular processes but as some antimicrobials have been shown to have anti-viral activity (Wu *et al.*, 2004) and as both were produced by only a single strain which significantly inhibited PSV entry without cytotoxicity, we hypothesized that these might be good NPs for further study.

Some cytotoxic strains that inhibited PSV infection also produced antimicrobials such as Valinomycin, Actinomycin D and Puromycin. Valinomycin, a naturally occurring dodecadepsipeptide involved in potassium transport and produced by *Streptomyces fulvissimus* (Huang *et al.*, 2021), was detected in the crude extracts of *Streptomyces* B1, PR5 and CW2- strains identified as cytotoxic with anti-PSV entry activity. However, as Valinomycin was found to have significant anti-viral activity against SARS-CoV (Wu *et al.*, 2004), it was deemed a potential NP for further analysis. Actinomycin D and Puromycin have been used in the treatment of different diseases. For example, Actinomycin D is a well-established chemotherapeutic agent (Liu *et al.*, 2016) and Puromycin has shown promise as an indicator of cancer progression when used as a radiopharmaceutical (Aviner,2020). Actinomycin D was also shown to have anti-viral activity (Guo *et al.*, 1998; Jeeninga *et al.*, 1998). Therefore, although the extracts were cytotoxic to cells the strains might still produce antimicrobial compounds, such as antimicrobials with potential anti-HIV activity. As we did not know the concentration of the antimicrobial in the crude extracts, we had to determine the minimum concentrations of Valinomycin, Antimycin, Puromycin and Actinomycin D that were not cytotoxic to TZM-bl cells, a modified reporter HeLa cell line.

Antimycin inhibits mitochondrial electron transport at complex III resulting in the collapse of the proton gradient across the mitochondrial inner membrane which decreases mitochondrial membrane potential (Ogita *et al.*, 2009). Lin *et al.* (2017) showed that murine osteoblasts incubated for 24 hours in the presence of 50 µM Antimycin showed significant cell death as well as Antimycin-induced apoptosis (Lin *et al.*, 2017). Additionally, Hytti *et al.* (2019) tested Antimycin at a minimum concentration of 20 µM, which also showed significant cell death (Hytti *et al.*, 2019). Puromycin acts by inhibiting protein synthesis by ribosome-catalyzed incorporation into the C-terminus of elongating chains, blocking extension and resulting in the complete termination of translation (Aviner,2020). Puromycin is non-selective and extremely cytotoxic, and therefore not used as a clinical antibiotic (Steitz,2008). Puromycin is typically used as a selection marker for cells engineered to express resistance transgenes and is also used a probe for protein synthesis across various cell-based models (Aviner,2020). On the other hand, Valinomycin is a potassium ionophore and has shown to induce apoptosis in numerous mammalian cell lines (Furlong *et al.*, 1998; Abdalah *et al.*, 2006). The cytotoxicity described in the literature was supported by our findings: all four antimicrobials showed significant cell death at concentrations up to 1 µg/mL. Further analysis found that the minimum concentration cytotoxic to TZM-bl cells varied according to antimicrobial: Actinomycin D: >6.4 nM, Valinomycin: >36 nM, Antimycin: >1.8 µM, Puromycin: >73 nM.

The IC_{50} was calculated using a range of antimicrobial concentrations below the cut-off values for cytotoxicity. The dose-response model did not fit the data for Antimycin and Valinomycin whereas the IC_{50} for Puromycin was termed ambiguous. This suggests that the range of antimicrobial concentration was not suitable to identify the IC_{50} for these antimicrobials. When the PSV inhibition data for Valinomycin and Antimycin were analyzed by non-linear polynomial regression it generated a non-monotonic graph. Non-monotonic graphs are defined when the slope of dose response curves change from negative to positive usually at low concentrations (Timsit *et al.*, 2022). This type of curve has been described for variants with drug resistant mutations to an RT inhibitor and it was suggested that the type of effect or mechanism of action of these drugs differ according to their concentration, resulting in a biphasic response (Zhang *et al.*, 2013). In the case of Antimycin and Valinomycin, it is possible that the antimicrobials' effect on PSV inhibition occurs at low concentration followed by cytotoxicity at higher doses. Alternatively, the antimicrobials might influence PSV inhibition via different mechanisms depending on concentration e.g., changing receptor concentrations vs inhibiting protein synthesis.

Interestingly, Valinomycin has been shown to directly inhibit the replication and budding of various viruses, including human coronaviruses, bunyaviruses, enteroviruses, and flavivirus (Zhang *et al.*, 2020). Studies have shown that Valinomycin displayed anti-MERS-CoV activity with an IC_{50} value as low as 5 nM in Vero E6 infected cells (Sandler *et al.*, 2020). Additionally, it was effective against human coronavirus (IC_{50} = 67 nM), Zika virus (IC_{50} = 78 nM), and five other viruses where the IC_{50} values ranged from 41 to 971 nM (Sandler *et al.*, 2020). It was suggested that Valinomycin acted by preventing the processing of viral Envelope glycoproteins (G protein), and thereby increasing its susceptibility to endo-β-N-acetylglucosaminidase H cleavage. Incomplete processing of the glycoprotein prevent its incorporation in the viral budding process (Pettit *et al.*, 1999). HIV-1 has an envelope glycoprotein (Env) which allows for virus binding to the CD4 receptor and CCR5 or CXCR4 co-receptors (depending on tropism) (Prabakaran *et al.*, 2007). PSVs only undergo a single round of infection due to the absence of the *env* gene in the packaged viral RNA and it is possible that PSVs are not the ideal assay to determine the inhibitory activity of NPs, especially when they target incorporation of Env in viral particles. Although, PSV have been used to screen for NPs with anti-viral activity (Q. Li *et al.*, 2018), it is possible that the inhibition assay should be repeated with live virus. Furthermore, a recent study found that the toxic effect of Valinomycin on host cells could be significantly reduced by incorporation of Valinomycin in liposomes (Daoud and Juliano, 1986). Infection by live virus of peripheral blood monocytes in the presence of Valinomycin delivered as liposomes could mimic more accurately HIV-1 infection, with the addition of reduced cytotoxicity. This approach might mitigate the biphasic dose response curve observed in this study.

Actinomycin D was shown to inhibit minus-strand transfer by preventing the activity of the nucleocapsid protein and hence HIV infection (Guo *et al*., 1998). It was the only antimicrobial with an IC_{50} (27 nM) for PSV inhibition derived from concentrations not cytotoxic to TZM-bl cells. However, this value was generated in the absence of maximum inhibition. When we repeated the dose response analysis including concentrations greater than the cut-off for cytotoxicity, we obtained an IC_{50} for Puromycin (67.7 nM) and Actinomycin D (9.15 nM). However, as these values were derived from concentrations most likely cytotoxic, it is possible the IC_{50}s are not accurate. Interestingly, Actinomycin D has been shown not to cause cell death in KLM-1 cells at 10 nM after 24, 72 and 96 hours (Liu *et al.*, 2016) although it is highly toxic to wild-type HeLa cells within the range 0.01-2.5 µg/mL, (Sawicki and Godman*.,* 1971). It is thus unclear whether the IC_{50} established in our study indicates inhibition of PSV infection. Both CC_{50} and IC_{50} values need to be determined across a wide range of concentrations to calculate the therapeutic index, a reliable measure of drug safety.

We included Anisomycin in the analysis because it was unique to a single strain with anti-viral, non-cytotoxic activity. However, we were unable to determine the range of Anisomycin concentration that was cytotoxic and instead extrapolated from previous values and tested IC_{50} from 3769 nM to 0.05 nM. Anisomycin displayed an EC_{50} of 35 µM against smooth muscle cells (Croons *et al.*, 2009). The $IC5_{50}$ we established was 61.6 nM, indicating that there should be no impact on cell death if used at this concentration in the PSV infection assay. However, the cytotoxicity of compounds is cell-specific and the IC_{50} was very similar to the CC_{50} established by Lee *et al.* (2005), suggesting that the inhibition seen was due to cytotoxicity. For future research we would include testing the cytotoxicity of Anisomycin at these lower concentrations, as this was not established in this study.

In conclusion, we have identified filamentous actinobacteria strains that produce compounds that inhibit PSV entry. Mass spectrometry identified nine known antimicrobials, and of these, five were tested for the ability to inhibit PSV infection. All the antimicrobials seemed to inhibit infection however, they were also cytotoxic to the reporter cell line. Actinomycin D and Anisomycin may have therapeutic value, but additional studies are needed to identify the therapeutic index and thus confirm their candidature as potential novel ARVs.

Chapter 4: The impact of *Gardnerella vaginalis* on HIV-1 infection

4.1 Introduction

The results of studies such as FACTS-001 and MTN 003 (VOICE) indicated that the efficacy of PrEP varied in its ability to reduce HIV-1 acquisition amongst women from Sub-Saharan Africa (Delany-Moretlwe *et al.*, 2018). This variability could be due to numerous factors, such as adherence (Van Der Straten *et al.*, 2012) and/or the diverse microbiota of the FGT (Hoang *et al.*, 2020). The incidence of BV is estimated to be 50% in Sub-Saharan Africa and, as it has been shown to be associated with increased acquisition of HIV-1, might be fueling the HIV pandemic in Africa (Javed *et al.*, 2019; Apalata *et al.*, 2021). The possible mechanisms include increased cervicovaginal inflammation due to the presence of non-commensal BVAB (Shannon *et al.*, 2017) or the breakdown of the cervicovaginal mucus barrier by virulence factors such as sialidase (Hoang *et al.*, 2020). Furthermore, BV has also been associated with reduced efficacy of ARVs in the FGT potentially due to microbial metabolism of TVF (Taneva *et al.*, 2018). It has been suggested that BVAB such as GV might produce compounds that trigger or facilitate the onset of BV and thereby, directly or indirectly, increase the chance of HIV infection (Shishpal *et al.*, 2020). However, GV is also present in BV-negative women (Janulaitiene *et al.*, 2017), suggesting that some strains might not be pathogenic because they either do not produce virulence factors or are unable to establish biofilms.

Recently, it has been shown that GV comprises of 12 genotypes subdivided across the newly described species: *Gardnerella leopoldii*, *Gardnerella piotii* and *Gardnerella swidsinskii* (Vaneechoutte *et al.*, 2019). Furthermore, when GV genotypes were compared to the absence and presence of virulence genes, it was shown that only certain genotypes carried genes for sialidase, suggesting that some GV strains were pathogenic whereas others were naturally occurring commensals (Tarracchini *et al.*, 2021). Therefore, when preliminary data generated in our lab showed that the ATCC strain of GV*,* which lacks a sialidase gene, seemed to inhibit HIV-1 infection, we hypothesized that perhaps this strain could protect against HIV infection through the production of anti-viral active secondary metabolite(s) and/or proteins. In this chapter, we investigated whether metabolites and proteins produced by GV had an inhibitory impact on HIV PSV entry.

4.2 Results

4.2.1 The effect of *G. vaginalis* on HIV-1 infection

GV was cultured and the cell-free preparation was added to the PSV infection assay to confirm previous findings that the bacteria released compounds in the culture medium that prevented HIV infection. Although the abiotic culture medium inhibited PSV infection by 67%, the BHI-only control reduced infectivity by 38%, suggesting that something in the culture medium was also affecting HIV infection (Figure 4.1). To separate the potential inhibitory compounds, the GV abiotic culture medium was fractioned by AS precipitation and each fraction was desalted by dialysis prior to concentration by freeze drying.

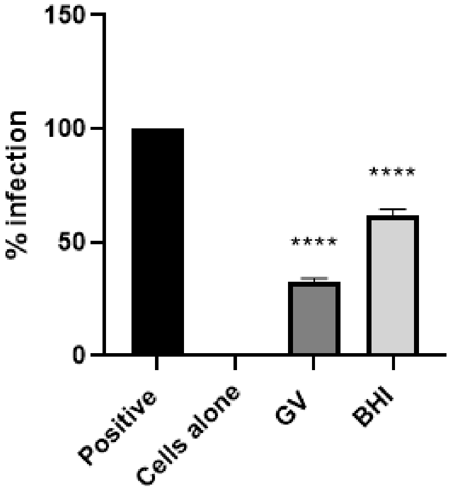

Figure 4.1: **The effect of culture medium on PSV entry**. BHI was cultured for 48 hrs with and without *G. vaginalis*. The culture medium was centrifuged and filter-sterilized. TZM-bl cells (5x10^4 cells/mL) were infected with 200 TCID$_{50}$ PSV with 5 µL of GV culture medium and 5 µL BHI medium and incubated for 48hrs at 37°C, 5% CO$_2$. Percentage infection [calculated by comparing the relative light units (RLU) of each infection relative to the RLU of a PSV only control (positive)]. The negative control (cells alone) is also shown. Bars represent the mean of two independent experiments with error bars indicating standard deviation. Statistical analysis was carried out using GraphPad one-way ANOVA and post-hoc Dunnett's multiple comparison test. *, **, *** and **** represent P<0.05, 0.01, 0.001 and 0.0001 respectively.

To determine whether the BHI-associated inhibitory factor could also be isolated, AS precipitation, dialysis and freeze-drying was also carried out on BHI. Only AS at 60, 70 and 80%

levels precipitated proteins from both GV abiotic culture medium and BHI. SDS-PAGE was performed to determine the presence/absence of protein after AS precipitation. The results showed that protein was present in the 60, 70 and 80% levels. A number of factors such as pH affect the efficiency of precipitation (Nooralabettu,2014) and this might have affected the ability to precipitate protein across all salt concentrations. Fractions at a final concentration of 20 µg were added to the PSV entry assay (Figure 4.2) and GV abiotic culture medium showed no significant inhibition at all salt levels. The BHI fractions, pelleted at 60% and 70% AS saturation, inhibited PSV infection by approximately 50%, albeit with no statistical significance (Figure 4.2A), similar to what was observed with the BHI medium before fractionation (Figure 4.1). Furthermore, none of the fractions affected cell viability (Figure 4.2 B). Overall, this suggested that a non-cytotoxic component of the BHI culture medium inhibited PSV infection. Unexpectedly, the abiotic GV culture medium had no effect on PSV infection, suggesting that salting out had not precipitated the inhibitory compound found in BHI nor that produced by the bacteria. It is possible that the presence of the bacteria could lead to the proteolytic degradation of the BHI-associated inhibitor so that its concentration was too low to isolate by AS precipitation.

Alternatively, GV growth has been shown to decrease the pH of culture medium from pH 8 to pH 4.6-6.0 (Mirmonsef *et al.*, 2016) which could inactivate the BHI- and GV-associated inhibitory factors and/or influence the efficiency of AS precipitation (Nooralabettu,2014). With regards to the GV-associated inhibitor, it is also possible that GV could be producing a small organic molecule not susceptible to precipitation by AS.

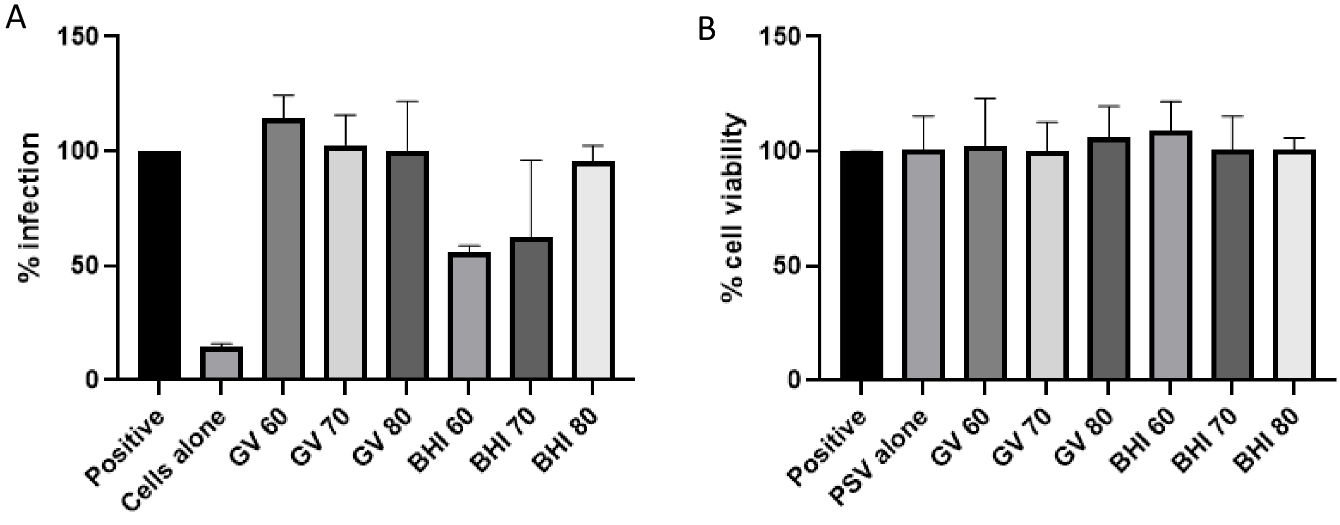

Figure 4.2 **The effect of 20 µg *G. vaginalis* protein on PSV entry and cell viability.** *G. vaginalis* was grown in BHI for 48hrs at 37°C. The cultures as well as BHI alone were then centrifuged, and an AS precipitation performed yielding the following precipitants: GV 60% AS; *G. vaginalis* 70% AS; GV 80% AS; BHI 60% AS; BHI 70% AS; BHI 80%. TZM-bl cells ($5x10^4$ cells/mL) were infected with PSV equivalent to 200 $TCID_{50}$ in the presence of 20 µg from each fraction and were incubated for 48hrs at 37°C, 5% CO_2. **(A)** Percentage infection (calculated by comparing the relative light units (RLU) of each infection relative to the RLU of a PSV only control (positive). The negative control (cells alone) is also shown. **(B)** An MTT assay was performed to determine the effects of 20 µg protein on TZM-bl cell viability. The percentage cell viability was calculated by dividing the fold-change in absorbance of each treatment to the fold change in absorbance of the cells only control (positive). Bars represent the mean of two independent experiments with error bars showing standard deviation. Statistical analysis was carried out using GraphPad one-way ANOVA and post-hoc Dunnett's multiple comparison test. *, **, *** and **** represent P<0.05, 0.01, 0.001 and 0.0001 respectively.

4.2.2. The effect of *G. vaginalis* active metabolites on pseudovirus entry and cell viability

4.2.2.1 Identification of a GV cell-associated bioactive compound

LLE is a common extraction method for natural products from microbial cultures and yields comparable results to other extraction methods (Schneider *et al.*, 2021). As AS did not apparently precipitate the GV-associated inhibitory factor, we hypothesized that GV might produce a small organic molecule that inhibited HIV infection. To test this hypothesis, LLE was carried out on the abiotic culture medium of GV strain ATCC 14018 to separate active metabolites based on their relative solubilities in organic solvents in decreasing polarity: methanol, ethyl acetate and hexane. This resulted in four separate fractions: 1) broth extracted with ethyl acetate, 2) cell pellet methanol wash, 3) cell pellet ethyl acetate wash, and 4) cell pellet hexane wash. In addition, BHI alone underwent LLE to determine the presence of any bioactive metabolites in the medium itself. Each of the fractions from GV (n=4) and BHI (n=1) were added to the PSV entry assay at final concentrations of 50 µg/mL and 25 µg/mL to determine which fraction contained anti-viral metabolites. The ethyl acetate GV cell mass wash significantly reduced PSV infection at 50 µg/mL and 25 µg/mL ($p <0.01$ and $p < 0.05$ respectively), with no significant impact on cell viability (Figure 4.3). This suggested that crude extraction with ethyl acetate had isolated a small cell-associated organic molecule with anti-HIV activity that was not released into the culture medium or was secreted but at concentrations too low to detect. Unexpectedly, ethyl acetate LLE of BHI at 50 µg/mL also showed a significant decrease in PSV entry without affecting cell viability (Figure 4.3 A and C). It is possible that BHI either contains both proteinaceous and secondary metabolites that inhibit PSV entry or LLE extraction enriched for the protein inhibitor fractionated by AS precipitation. It has been reported that LLE extraction method can also isolate proteins (Yeung and Stanley,2011). However, as we identified a fraction from GV cells with anti-HIV activity, we continued to enrich for the activity using SPE.

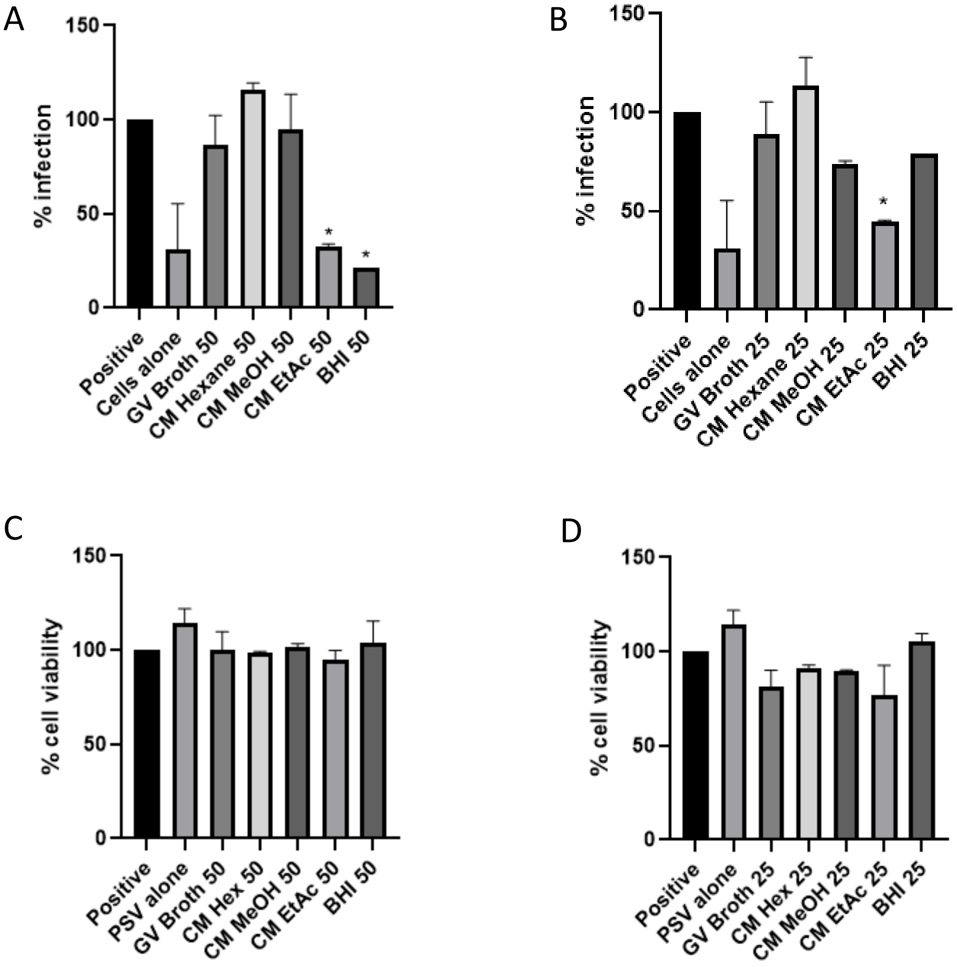

Figure 4.3: **The effect of *G. vaginalis* post LLE on PSV entry.** GV was cultured in 50 mL BHI for 48hrs. Four cultures were then pooled to a total volume of 200 mL and centrifuged. LLE was performed resulting in four fractions: GV broth; GV cell mass Methanol wash; GV cell mass Ethyl Acetate wash and GV cell mass Hexane wash. TZM-bl cells (5x10^4 cells/mL) were infected with 200 $TCID_{50}$ PSV in the presence of either **(A)** 50 µg/mL and **(B)** 25 µg/mL of either fraction and incubated for 48hrs at 37°C, 5% CO_2. Percentage infection (calculated by comparing the relative light units (RLU) of each infection relative to the RLU of a PSV only control (positive). The negative control (cells alone) is also shown. An MTT assay was performed to determine the effect of **(C)** 50 µg/mL and **(D)** 25 µg/mL of either fraction on TZM-bl cell viability. The percentage cell viability was calculated by dividing the fold-change in absorbance of each treatment to the fold change in absorbance of the cells only control (positive). Bars represent the mean of two independent experiments with error bars showing standard deviation. Statistical analysis was carried out using GraphPad one-way ANOVA and post-hoc Dunnett's multiple comparison test. *, **, *** and **** represent P<0.05, 0.01, 0.001 and 0.0001 respectively.

SPE, using a C18 column, was used to fractionate the GV cell mass ethyl acetate sample into three fractions labelled SPE 1, SPE 2 and SPE 3. The addition of 50 µg/mL and 25 µg/mL of each of the fractions to the PSV entry assay showed that SPE 1 displayed significant inhibition at 50 µg/mL and 25 µg/mL ($p < 0.0053$ and $p < 0.0078$ respectively), whereas SPE 2 and SPE 3 showed no significant inhibition of the PSV (Figure 4.4 A and B). None of the fractions (SPE 1, SPE 2 or SPE 3) impacted cell viability (Figure 4.4.C and D). The ability of SPE 1 at 50 µg/mL and 25 µg/mL to inhibit PSV entry suggested that this fraction could comprise an anti-viral metabolite that does not affect cell viability. Therefore, SPE 1 was further analyzed by mass spectrometry using a QTOF followed by GNPS database screening to identify any known metabolites that could explain the anti-viral properties seen.

Mass spectrometry and GNPS analysis identified two compounds (Table 4.1) that might potentially contribute to the inhibition of PSV infection by SPE1. Dexpanthenol is an alcoholic analogue of D-pantothenic acid and a member of the vitamin B complex. It is a precursor of co-enzyme A, an important factor in the metabolism of carbohydrates, fatty acids, proteins, and steroid hormones (Ebner *et al.*, 2002).

Table 4.1 Identification of putative GV secondary metabolites with anti-viral properties

Fraction	**Identified compound**	**Cosine score**	**Mass difference**
SPE Fraction 1	Dexpanthenol	0.84	0.001
	Pyridoxine/Pyridoxol	0.9	0.082

There is evidence that pantetheine might inhibit SARS-CoV-2 infection but there has been no such evidence regarding HIV (Cit *et al.*, 2022). Pyridoxine is commonly known as Vitamin B_6. Vitamin B_6 naturally appears as pyridoxine, pyridoxal and pyridoxamine. They are enzymatically interconvertible, and are all phosphorylated to pyridoxal-5-phosphate (PLP) via a phosphokinase (pyridoxal kinase) (Van Der Watt *et al.*, 2011). PLP is the biologically active form of vitamin B_6 and this compound has been identified as an inhibitor of HIV-1 Int (Williams *et al.*, 2005) although there is no evidence to suggest that pyridoxine inhibits HIV infection. *Actinobacteria* do produce

Vitamin B_6 starting from glyceraldehyde-3-phosphate and d-ribulose 5-phosphate (Yoshii *et al.*, 2019) but there has been no reports suggesting that GV produces Vitamin B_6.

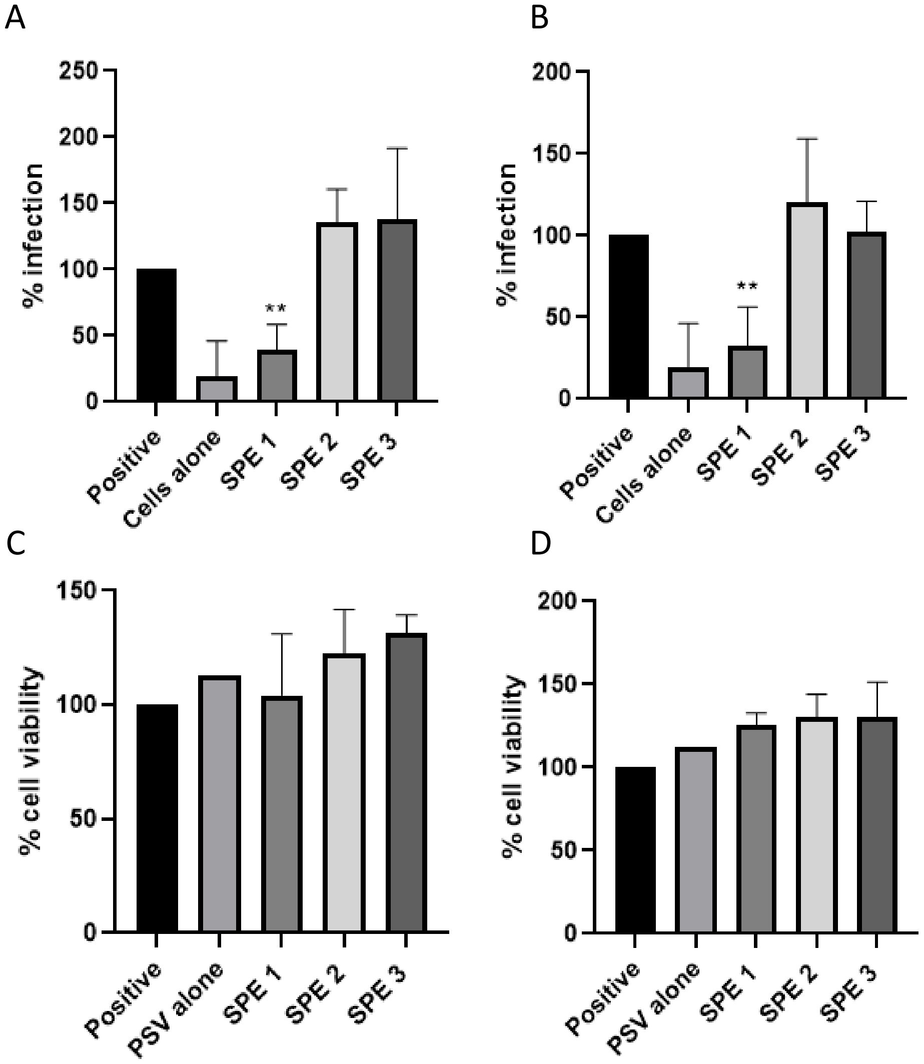

Figure 4.4: **The effect of the ethyl acetate *G. vaginalis* extract post SPE on PSV entry and cell viability**. Solid Phase Extraction (SPE) was used to fractionate the active GV sample. The resulting fractions were SPE 1, SPE 2 and SPE 3 and were extracted in 2 mL methanol, 2 mL acetonitrile and 2 mL hexane respectively. TZM-bl cells ($5x10^4$ cells/mL) were infected with 200 $TCID_{50}$ in the presence of either **(A)** 50 and **(B)** 25 µg/mL of either fraction and incubated for 48hrs at 37°C, 5% $CO_{2.}$ Percentage infection (calculated by comparing the relative light units (RLU) of each infection relative to the RLU of a PSV only control (positive). The negative control (cells alone) is also shown. An MTT assay was performed to determine the effect of **(C)** 50 and **(D)** 25 µg/mL of either fraction on cell viability. The percentage cell viability was calculated by dividing the fold-change in absorbance of each treatment to the fold change in absorbance of the cells only control (positive). Bars represent the mean of two independent experiments with error bars indicating standard deviation. Statistical analysis was carried out using GraphPad unpaired t-test and one-way ANOVA and post-hoc Dunnett's multiple comparison test *, **, *** and **** represent P<0.05, 0.01, 0.001 and 0.0001 respectively.

As LLE of BHI also enriched for anti-HIV activity, we wanted to determine whether Dexpanthenol and/or Pyridoxine was present in the LLE fraction. When SPE was carried out on the BHI LLE fraction, neither compound was present, suggesting that the anti-viral activity associated with BHI medium was not due to Dexpanthenol and/or Pyridoxine.

4.3 Discussion

Bacterial Vaginosis has been found to increase susceptibility to HIV-1 infection through inflammation, disruption of the mucosal barrier in the FGT and by hindering the efficacy of ARVs (Atashili *et al.*, 2008; Klatt *et al.*, 2017). GV is known to be associated with BV however, its exact role in the pathology is still unclear especially given the newly discovered genotypic and phenotypic diversity of the genus (Vaneechoutte *et al.*, 2019). The ability of certain species of GV to produce sialidase, which can reduce the protective properties of the mucosal barrier in the FGT, is a possible mechanism of GV pathogenesis (Moncla *et al.*, 2016). Additionally, the production of Vaginolysin and the ability of GV to form biofilms could also contribute to BV progression (Alves *et al.*, 2014; Shishpal *et al.*, 2020).

Surprisingly, data generated in our laboratory, and confirmed here, suggested that GV ATCC 14018 inhibited PSV entry of a reporter cell line. *Gardnerella* spp. are diverse, with recent studies suggesting that the variation in phenotype amongst the specific strains could explain possible pathogenesis of BV (Janulaitiene *et al.*, 2017). Some *Gardnerella* spp. produce sialidase which degrades mucin (Schellenberg *et al.*, 2016) and VLY, a cholesterol-dependent cytolysin (Yeoman *et al.*, 2010) that target and lyse human cells via the human complementary regulatory molecule (hCD59) and induce IL-8 production in human epithelial cells (Gelber *et al.*, 2008). Differential expression of these virulence determinants might determine whether some GV strains are pathogenic or harmless microbes of the FGT. Although there have been reports on how sialidase might enhance HIV infection, no potential mechanism for VLY has been described. GV ATCC 14018 which does not produce sialidase, produces VLY (Randis *et al.*, 2013) which could inhibit HIV infection by lysing host cells. HeLa cells have been shown to express CD59, the molecule that renders cells susceptible to VLY mediated lysis (Gelderman *et al.*, 2002). It is thus possible that

TZM-bl cell lysis in the presence of GV ATCC 14018 abiotic culture medium is responsible for the apparent inhibition of PSV infection observed *in vitro*.

When AS precipitation was carried out on GV abiotic culture medium, it did not enrich for anti-PSV infection activity, suggesting that GV was not secreting a proteinaceous compound into the culture medium with the potential to inhibit HIV infection. This also suggested that either AS failed to precipitate VLY or it was not the compound responsible for the anti-viral activity. AS of BHI did result in the enrichment of anti-viral activity, suggesting that the inhibition of PSV infection observed by GV abiotic culture medium could be due to a component of the culture medium itself. However, AS of the GV abiotic culture medium did not isolate the same activity and we hypothesized that bacterial growth might have affected the components of BHI, such as proteolytic degradation, leading to a loss of HIV infectivity. Alternatively, it is possible that the precipitation method needed optimization as pH can influence the crude purification method (Nooralabettu,2014). AS precipitation is a common yet crude protein extraction method and has been used to isolate a number of proteins from bacterial cultures. For example, a protease from *Bacillus cereus* TD5B was extracted using this method (Winarti *et al.*, 2018). However, a disadvantage of AS precipitation is that contaminants often precipitate with the protein/s of interest. Furthermore, AS can lead to loss of protein and it was shown that purification of Papain was more efficient when AS was not used (Purwanto,2016). Further purification, such as ion exchange chromatography and gel filtration might be better suited for purification of the inhibitor from the GV abiotic culture medium (Duong-Ly and Gabelli, 2014). We are thus unable to confirm whether GV secretes a compound into the culture medium with anti-viral activity or whether the activity is due to a component of the BHI media without further purification optimization.

After finding that AS of GV abiotic culture medium did not enrich for an anti-viral protein, we conducted LLE on the abiotic culture medium as well as the cell mass to determine whether GV produced an anti-viral secondary metabolite. The results showed that after washing the cell mass with ethyl acetate, the fraction contained a cell-associated compound that inhibited PSV infection in our reporter cell line at 50 µg/mL and at 25 µg/mL without impacting cell viability. We further fractionated the sample using SPE to improve sample preparation, achieve higher selectivity and ensure precise compound detection (Abd-Talib *et al.,*2014). The principal is similar to LLE and involves the separation of solutes between two phases, however in SPE, the separation is between a liquid which is the sample matrix/solvent with the analyte and a solid phase which is the column/cartridge (Abd-Talib *et al.,* 2014). After performing the SPE with methanol, acetonitrile and hexane washes, the fraction eluted from the methanol wash was the only fraction that showed significant inhibition against PSV infection at 50 µg/mL and at 25 µg/mL. This indicated that a possible cell-associated, mid-polar, anti-viral metabolite was produced by GV that did not impact cell viability.

This fraction was then analyzed by mass spectrometry and screened against the GNPS database to identify the anti-viral metabolite. GNPS identified 2 compounds Dexpanthenol and Pyridoxine that are components of Vitamin B_6 complex . A potential mechanism for the effect of Vitamin B_6 on HIV infection might be a result of glucocorticoid-mediated expression of HIV replication. The HIV-1 long terminal repeat (LTR) promoter contains glucocorticoid-responsive element (GRE)-like sequences and therefore glucocorticoids stimulate viral replication. Vitamin B_6 inhibits glucocorticoid receptor mediated gene expression (Allgood *et al.,* 1990) and thus potentially HIV replication. Therefore, GV could produce pyridoxine *in vitro* which is taken up by the reporter cell line and activated by phosphorylation to form Vitamin $B_{6.}$ Levels of Vitamin B_6 could increase, preventing the glucocorticoid receptor from activating the LTR and thus Tat protein (Trans-activator of Transcription) activity, ultimately decreasing luminescence. However, we did not detect Vitamin B_6 in the abiotic culture medium which could suggest that it is not secreted or that LLE of the abiotic culture medium was not robust enough to isolate the secondary metabolite. Future experiments could include checking whether Vitamin B_6 has any effect on PSV infection *in vitro*.

Although no previous studies have investigated the NPs or metabolites produced by GV specifically in the context of BV, many studies have investigated the role of metabolites as potential biomarkers in the diagnosis and treatment of BV. Srinivasan *et al.* (2015) used a global metabolomics approach to investigate the differences in metabolic composition and concentrations associated with BV. Overall, this study looked at the cervicovaginal lavage fluid from women with and without BV and found that women who were BV-positive had metabolite profiles marked by lower concentrations of amino acids and dipeptides while having high levels of amino acid catabolites and polyamines. Additionally, they found metabolic signatures associated with the presence and concentration of specific bacteria (Srinivasan *et al.*, 2015). For example, GV was found to be positively correlated with increased *N*-acetylaspartate, 12-hydroxyeicosatetraenoic acid, and fatty acids such as eicosenoate and dihomo-linoleate (Srinivasan *et al.*, 2015). Yeoman *et al.* (2013) also established the metabolomic profiles of lavage samples from women with and without BV. The profiles revealed two distinct symptomatic BV types with each type correlating to the presence of different bacterial taxa and metabolites. Interestingly, *Gardnerella* spp. was positively correlated with the presence of diethylene glycol however no reason for the link was investigated (Yeoman *et al.*, 2013). It might be interesting to check whether *N*-acetylaspartate, 12-hydroxyeicosatetraenoic acid, eicosenoate, dihomo-linoleate and diethylene glycol have an impact on HIV infection.

Although we identified anti-viral activity associated with GV cells and the abiotic culture medium inhibited PSV infection, we also found that BHI medium used to culture GV also inhibited PSV infection albeit to a lesser degree. We thus carried out the same purification techniques on BHI as we did for the abiotic culture medium and cell mass. We found that AS precipitation and combined LLE and SPE extraction apparently enriched for inhibitory activity from BHI. This suggests that BHI components seem to be responsible for the inhibition observed for the abiotic culture medium. The LLE and SPE enriched for activity in the GV cell mass, and this was identified as components of the Vitamin B_6 complex. These compounds were absent in BHI suggesting that they are unique to GV. However, this hypothesis requires further study as the purification methods and treatment of the samples have to be optimized to confirm that Vitamin B_6 is produced by GV and not present in BHI and that Vitamin B_6 does inhibit PSV infection. It is possible that the Vitamin B_6 produced by GV is a by-product of metabolism when cultured in BHI. It is also possible that other metabolites were responsible for these results and that the GNPS library was unable to identify them, or they are novel compounds. Further work is required to determine this. Dexpantheol and Pyridoxine/Pyridoxol reference standards should be obtained and tested to determine if the activity matches the active fractions extracted from the ATCC strain of GV. Future studies should also include investigating whether different *Gardnerella* spp. such as *G.*

leopoldii, G. swidsinskii and *G. piotii*, also inhibit PSV infection and if so, these strains should be screened to identify those proteins and/or secondary metabolites. Despite the caveats of this study, it is the first to investigate whether NPs produced by GV could directly protect against HIV infection to identify potential ARV alternatives.

Chapter 5: Conclusion

Bacteria have been a source of NPs for more than 90 years and many of these have anti-viral activity (Omura *et al.*, 2001; Chen *et al.*, 2018; Singh, P *et al.*, 2019). NPs are a resourceful tool in developing novel treatments, especially in the context of HIV-1, which is an ongoing epidemic despite the establishment of HAART. The overarching aim of this project was to identify NPs produced by actinobacteria and GV with anti-HIV activity. Actinobacteria was chosen because they are well known sources of anti-microbial NPs whereas GV is associated with BV which has been shown to increase the acquisition of HIV. Of the 23 actinobacteria strains screened, 3 strains (*Streptomyces* strain #15, *Micromonospora* strain M8 and *Nocardia* strain B10 Fri) displayed anti-viral inhibition with minimal impact on cell viability. Further analysis of the extracts revealed that these strains produced known antimicrobials, used in the treatment of other diseases: Actinomycin D, Valinomycin, Puromycin, Antimycin and Anisomycin. However, none of the 5 antimicrobials seemed to significantly inhibit PSV infection within the range of antimicrobial concentrations that were not cytotoxic. Despite this, the protocol of screening filamentous actinobacteria for NPs could be promising in the search for novel, anti-HIV treatments and this was the first time filamentous actinobacteria were screened against this PSV assay. However, future studies should investigate the cytotoxicity of antimicrobials at lower concentrations, as this was not established in this stud Additionally, using genome-mining on filamentous actinobacteria allows for the exploration of novel antimicrobial gene clusters which could potentially display antiviral activity (Singh,T *et al.*, 2021). Another aspect to consider for future studies would be the synergistic effect of multiple antimicrobials, and in conjunction with ARVs.

When our lab determined that GV culture medium inhibited HIV infection we aimed to identify the potential inhibitory compound. Therefore, the second objective of this study was to determine whether GV, a BVAB, produced proteins or secondary metabolites that inhibited PSV infection. We found that not only did the GV supernatant have anti-PSV infection activity, but that BHI also seemed to have similar activity albeit at lower levels. After crude protein extraction by AS precipitation, we found that GV did not produce an anti-viral protein but the BHI fractions seemed to have anti-HIV activity. Further purification techniques need to be carried out to confirm this finding.

We next tested whether GV might produce secondary metabolites using LLE and SPE to isolate the inhibitory compound from the GV supernatant as well as the cell mass. We found that GV produced a mid-polar fraction that significantly inhibited PSV infection without impacting cell viability and that this activity was not present in the abiotic culture medium. However, once again we also identified activity in BHI. However, as we did not detect activity in the GV abiotic culture medium, we analysed the cell mass SPE fraction on the mass spectrometer and GNPS database and identified Dexpanthenol and Pyridoxine, both of which are members of the Vitamin B_6 complex. When we did the same analysis on the BHI SPE fraction, we did not identify these compounds. We cannot exclude the possibility that the inhibition of PSV infection observed for the GV abiotic culture was not due to components of the BHI medium. Future studies would include exploring the use of different media types for *G. vaginalis* and seeing if bioactivity is observed or whether BHI contains components that act as an inducer for secondary metabolite production in *G. vaginalis*. The anti-PSV activity associated with GV cell mass requires further investigation, and as the genome sequence for ATCC14018 is known, this could allow for the identification of potential NPs that display antiviral activity. Therefore, this study found that GV produced Vitamin B_6 and that it may be responsible for the antiviral activity observed in this study.

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