Anti-HIV activity of the human antimicrobial peptide LL-37, and its engineered peptide, 17BIPHE2

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Abstract

Unwanted pregnancies and sexually transmitted infections (STIs) are major health concerns of women worldwide. These concerns have prompted efforts to develop Multipurpose Prevention Technologies (MPTs), which simultaneously provide contraception and prevent STIs, including HIV. LL-37, the only human cathelicidin and an effective spermicide on human sperm, has broad antimicrobial activity including in vitro activity against HIV. 17BIPHE2 is a truncated LL-37 peptide, engineered to contain 5 unnatural residues, thus limiting its protease degradation within vaginal fluid. Hence, this antimicrobial peptide (AMP) represents a promising MPT agent. It was therefore hypothesized that these peptides would be inhibitors of HIV infection in cell lines, PBMC, and CD4+ T cells. In the chronically infected ACH-2 cell line, there was significant reduction in p24 production when cells were treated with 17BIPHE2, but not LL-37. When 17BIPHE2 was pre-incubated with HIV prior to infection and present during infection, viral replication decreased in the TZM-bl reporter cell line, but this result was not recapitulated in the primary activated cells, PBMCs nor isolated CD4+ T cells. Conversely, pre-incubation of 17BIPHE2 with target cells prior to infection significantly inhibited HIV infection in a dose-dependent manner. Therefore, 17BIPHE2 may act on the cell or on the virus/cell interaction rather than on the virus itself to inhibit HIV infection.

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List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
ALU	Arbitrary luminescence units
AM	Acetoxymethyl
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
APC	Allophycocyanin
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CCL3	C-C chemokine ligand 3
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CVS	Cervicovaginal secretion
CXCR4	C-X-C chemokine receptor type
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluoroscein isothiocyanate

FPRL-1	Formyl peptide receptor-like 1
GM3	Monosialodihexosylganglioside
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HLA-DR	Human leukocyte antigen – DR isotype
IFN	Interferon
IL-12	Interleukin 12
IL-2	Interleukin 2
IL-8	Interleukin 8
JC-1	5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide
mDC	Monocyte-derived dendritic cell
mLC	Monocyte-derived Langerhans cell
MPT	Multipurpose prevention technology
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NMR	Nuclear magnetic resonance spectroscopy
P2X7R	Purinergic channel receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde

PHA	Phytohaemagglutinin
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute 1640 medium
RSV	Respiratory syncytial virus
TGF	Transforming growth factor
TNF	Tumour necrosis factor
V3	Variable loop 3
WHO	World Health Organization
ZIKV	Zika Virus

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Chapter 1: Introduction

1.1 Human immunodeficiency virus infection

Acquired immunodeficiency syndrome (AIDS) is characterized by a stark loss in CD4+ T cells, severe immunosuppression, and susceptibility to opportunistic infections and malignancies [1]. The disease develops as a result of progressive human immunodeficiency virus (HIV) infection without treatment. The primary target of HIV is CD4+ T lymphocytes, though the virus can also infect other cells that express the CD4 surface receptor. This includes tissue resident macrophages and dendritic cells [2]. HIV is primarily transmitted sexually through cell-associated or free virus in semen or cervicovaginal fluid. The virus can then enter mucosal tissue through small tears in the stratified squamous epithelium that might appear during intercourse [3]. Infection of target cells disseminates first with endocytosis by epidermal Langerhans cells which further leads to cell-associated transfer to other target cells. Productive infection thus occurs when the HIV envelope protein, gp120, interacts with CD4 resulting in a conformational change exposing the binding site for HIV co-receptors, C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) [3,4]. Whether the virus interacts with CCR5, CXCR4, or both, is referred to as viral tropism and it is dictated by the variable loop 3 of gp120 (V3) [5]. These interactions allow for fusion between the viral membrane and the cell membrane. Viral RNA then enters the cytoplasm where it can be transcribed into DNA by HIV reverse transcriptase, which can then enter the nucleus and integrate into the host genome via HIV integrase. HIV genes can then be transcribed and subsequently translated into viral proteins and virion assembly proceeds. HIV protease then processes viral pro-proteins and mature virions can bud from the cell [6] (Figure 1).

1.2 Young women and HIV

Young women are now the primary population affected by HIV, with almost 20 million women infected worldwide [7]. Globally, AIDS-related illnesses are one of the leading causes of death among women aged 15-49 years, with similar mortality rates to that of complications during childbirth [8]. In areas most affected such as Sub-Saharan Africa, the rate of new infection is up to three per 100 person years [9,10].

New infections continue to mount despite established preventative measures. Established approaches to prevent HIV infection include oral medications such as Truvada (tenofovir and emtracitabine, two nucleoside reverse transcriptase inhibitors) prophylaxis, referred to as pre-exposure prophylaxis or PrEP [11]. However, the need to consistently consume this medication for an extended time period makes the use of Truvada inaccessible and unappealing specifically for young women [11,12]. Other preventative agents are in development including dapivirine (non-nucleoside reverse transcriptase inhibitor) formulated into a silicone ring to be administered vaginally [13,14]. This dapirvirine ring was however only effective in reducing the risk of HIV infection by 27%–35% in phase III clinical studies [13,14]. There are also long-acting injectable agents which have shown potential but have not been approved [15]. Altogether this indicates an imminent need for novel anti-HIV preventative agents.

1.3 Anti-HIV antimicrobial peptides

Looking to pre-existing compounds with broad-spectrum antimicrobial activity such as antimicrobial peptides (AMPs) is one strategy employed in developing new HIV therapies or prophylactic agents. AMPs are small cationic and amphipathic peptides (<10 kDa) that are part of the host's innate immune defense against invading bacteria, viruses, fungi and protozoal parasites

[16,17]. They are expressed by all life forms including bacteria, plants, and animals including humans. The microbicidal activity of AMPs usually occurs by triggering membrane permeabilization. First, a strong electrostatic interaction occurs between the cationic amino acids on the AMP and anionic lipids on microbial cell membranes. Hydrophobic residues of the AMP and microbial lipid bilayers then also interact, subsequently leading to membrane perforation, loss of cell homeostasis, and finally cell death [18–20]. AMPs are selective toward microbes such as bacteria due to the presence of anionic lipids on the bacterial cell membrane, which are not present on eukaryotic cells, as well as the lack of cholesterol on bacterial cell membranes[21]. Cholesterol provides rigidity to eukaryotic membrane lipid bilayer thus providing protection from membrane disruption[21].

There is an existing extensive database (http://aps.unmc.edu/AP) which includes over 100 naturally occurring AMPs annotated for their anti-HIV activity [22]. Some of these peptides are approved for clinical use though are not necessarily used for their anti-HIV activity. For example, the FDA-approved antibiotic Gramicidin, isolated from *Bacillus brevisin,* also has spermicidal properties and exhibits anti-HIV activity in cervical explants [23,24]. The majority of the catalogued AMPs come from insects and amphibians [25,26] many of which have been screened for their anti-HIV activity [22]. Caerin, an amphibian AMP, has displayed anti-HIV activity through disrupting the HIV envelope [27]. However; gramicidin induces hemolysis of human red blood cells, indicating that cell permeabilization by AMPs may not be exclusive to microbes. This hemolysis was notably through a permeabilization mechanism atypical of most AMPs [28,29]. Such non-human AMPs may still pose other issues as anti-HIV agents. Since these are foreign peptides, antibody production may occur in humans. Therefore, searching for anti-HIV activity in human AMPs might be more efficacious.

There are two families of human AMP, defensins and cathelicidins, both of which have been investigated for anti-HIV activity. Alpha-defensins and beta-defensins exhibit their HIV inhibition by both acting directly on the virus and changing target cells to make them less susceptible to infection [30–33]. Anti-HIV effects have also been displayed by θ-defensins, or retrocyclins, but such peptides are only expressed by non-human primates [31,34,35]. Conversely, some defensins have also been reported as HIV infection enhancers [36]. Moreover, defensins contain several cysteine residues which make them challenging to chemically synthesize [22]. Cathelicidins, the remaining family of human AMPs, have thus been further targeted for their anti-HIV properties (see more below).

1.4 Introduction to LL-37

In humans, the most well studied form of cathelicidins is LL-37, which is processed from its propeptide, hCAP-18. Other processed forms include ALL-38 (LL-37 with an additional alanine in the N-terminus) and FALL-39 (LL-37 with two additional amino acids, phenylalanine and alanine, in the N-terminus)[37]. Immune cells and cells that are exposed to the external environment such as neutrophils, monocytes, mast cells, and epithelial cells are capable of expressing hCAP-18 [37,38]. Upon encountering infectious or inflammatory stimuli, hCAP-18 is converted into LL-37 [37,38]. LL-37 has microbicidal activity against over 50 bacterial species, 10 viruses and one yeast species, *Candida albicans* [39,40]. Analysis of the peptide structure by 3D nuclear magnetic resonance spectroscopy (NMR) displays that when LL-37 interacts with lipid bilayers, similar to structures in the bacterial cell membrane, LL-37 becomes an alpha helical amphipathic structure with one side of the helix enriched in cationic amino acids and the other side enriched in hydrophobic residues [40,41] (Figure 2). This positioning of these residues enables membrane permeabilization via LL-37 [20]. Its cationic face can interact with anionic head groups

of phosphatidylglycerols and cardiolipins in the bacterial cell membrane (Figure 3A). The hydrophobic residues of LL-37 then interact with the bacterial lipid bilayers (Figure 3B). Additional LL-37 peptide molecules are then recruited through the hydrophobic interaction between adjacent LL-37 peptides (Figure 3C). This leads to pore formation in the bacterial cell membrane and subsequent loss of bacterial homeostasis-leading to cell death [20,42,43]. The helical structure of LL-37 is, however, bent at serine 9, which divides the peptide into two domains: The *N*-terminal helix and the central helix. The central helix is essential to the peptide's microbicidal activity [17,40].

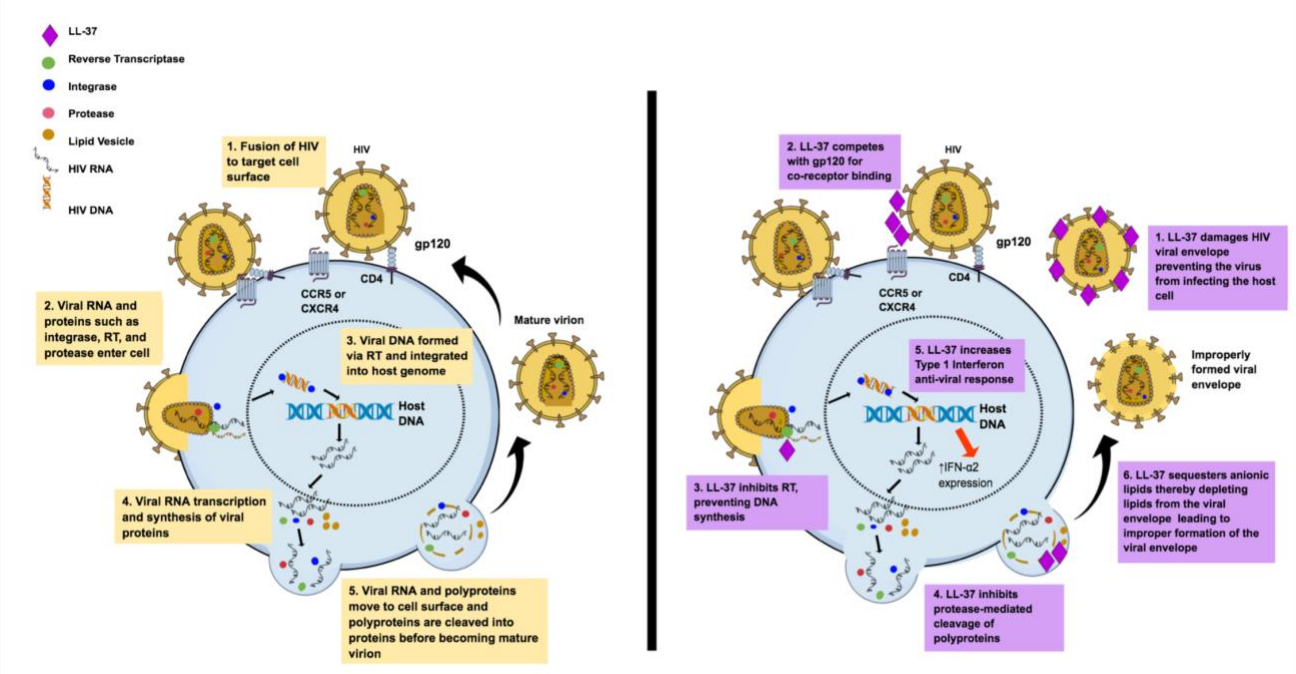

LL-37
Reverse Transcriptase
Integrase
Protease
Lipid Vesicle
HIV RNA
HIV DNA
1. Fusion of HIV to target cell surface
HIV
gp120
CD4
CCR5 or CXCR4
2. Viral RNA and proteins such as integrase, RT, and protease enter cell
3. Viral DNA formed via RT and integrated into host genome
Host DNA
Mature virion
4. Viral RNA transcription and synthesis of viral proteins
5. Viral RNA and polyproteins move to cell surface and polyproteins are cleaved into proteins before becoming mature virion
2. LL-37 competes with gp120 for co-receptor binding
HIV
gp120
CD4
CCR5 or CXCR4
1. LL-37 damages HIV viral envelope preventing the virus from infecting the host cell
5. LL-37 increases Type 1 Interferon anti-viral response
Host DNA
↑IFN-α2 expression
Improperly formed viral envelope
3. LL-37 inhibits RT, preventing DNA synthesis
6. LL-37 sequesters anionic lipids thereby depleting lipids from the viral envelope leading to improper formation of the viral envelope
4. LL-37 inhibits protease-mediated cleavage of polyproteins

Figure 1. Possible mechanisms LL-37 utilizes to interfere with the HIV life cycle

Reprinted from: Vera-Cruz A, Tanphaichitr N, Angel JB. Antimicrobial peptide, LL-37, and its potential as an anti-HIV agent. *Clin Investig Med*. 2021;44(3). Left panel denotes the normal life cycle of HIV and the right panel shows possible mechanisms by which LL-37 interferes with HIV. 1) LL-37 damages the viral envelope, 2) competition with gp120 for binding to CXCR4 or CCR5, 3) inhibition of HIV reverse transcriptase thus inhibiting DNA synthesis, 4) inhibition of HIV protease, which would prevent cleavage of polyproteins into functional proteins, 5) increasing the Type 1 Interferon anti-viral response, and 6) sequestering anionic lipids including gangliosides of the infected host cell during HIV replication thereby depleting the viral envelope of these lipids.

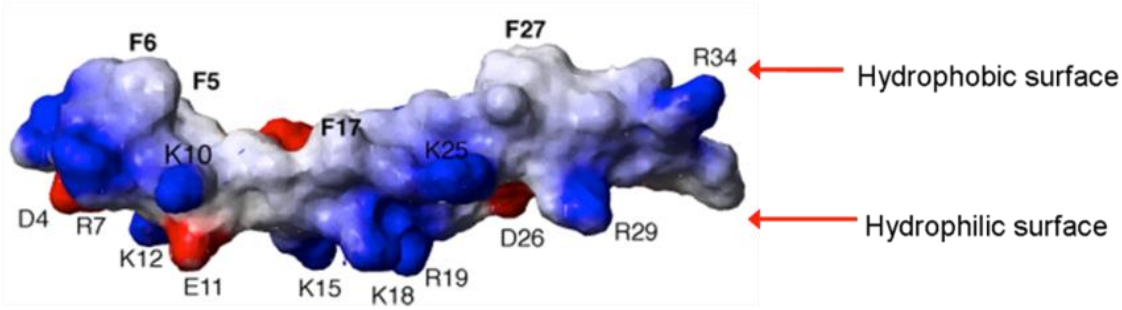

F6
F5
F27
R34
F17
K10
K25
D4
R7
K12
E11
K15
K18
R19
D26
R29
Hydrophobic surface
Hydrophilic surface

Figure 2. Structure of LL-37

Reprinted from: Wang G. Structures of human host defense cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles. *J Biol Chem*. 2008 Nov 21;283(47):32637-43. Cationic hydrophilic amino acids (blue color) localize on one face of the LL-37 alpha helix, whereas hydrophobic residues (red color) reside on the other face. LL-37 is bent at Serine 9, dividing the peptide into two domains the *N*-terminal helix and the central helix, the latter of which is essential for microbicidal activity.

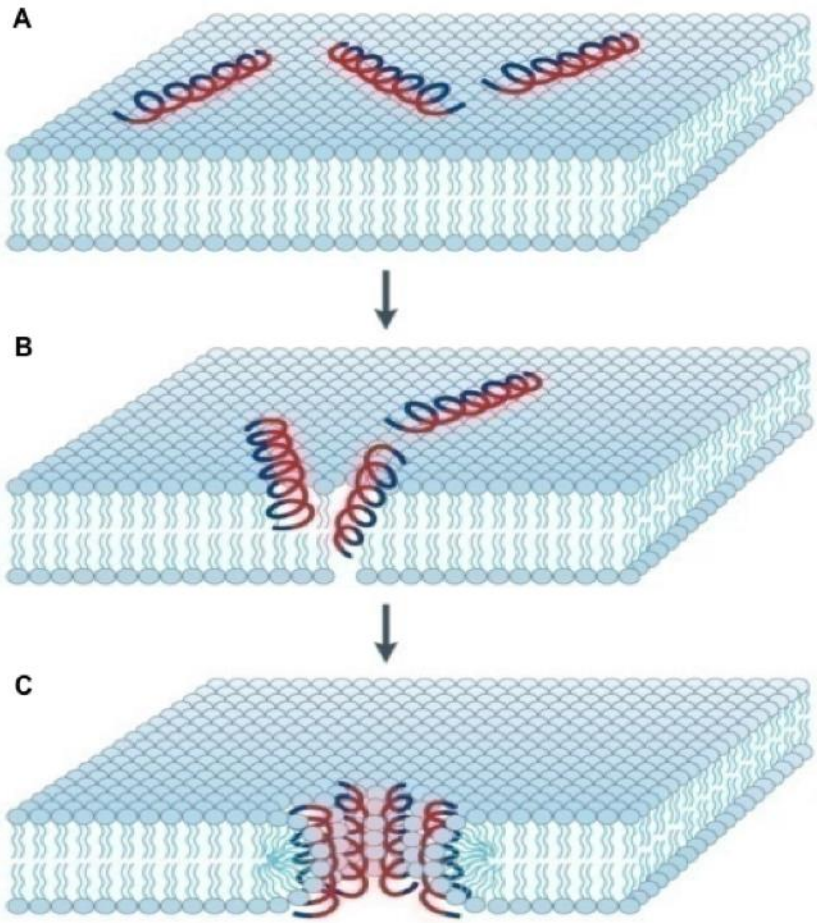

A
B
C

Figure 3. Mechanism of microbial cell membrane perforation by LL-37

Reprinted from Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?. *Nat Rev Microbiol.* 2005 Mar;3(3):238-50. A) The cationic side of LL-37 interacts with anionic head groups in the bacterial cell membrane. B) The hydrophobic residues of LL-37 interact with the bacterial lipid bilayers. C) LL-37 molecules are recruited through interaction with adjacent LL-37 hydrophobic residues. This leads to pore formation in the bacterial cell membrane and subsequent loss of bacterial homeostasis-leading to cell death.

As for the antiviral activity of LL-37, the mechanisms are not as clear. LL-37 has the ability to directly impact some viruses, such as influenza A and respiratory syncytial virus (RSV), to reduce the infectivity [44–46]. In other viral infections, LL-37 can modulate the susceptibility of the host cell to the virus. In Zika virus (ZIKV) a truncated LL-37 peptide, GF-17, inhibited infection of target cells by both its direct effect on the virus as well as changing the susceptibility of the host cell [45]. For the latter, LL-37 increased expression of the type I interferon, IFN-α2, in fetal astrocytes, allowing for the host cells to more efficiently respond to the virus [45]. LL-37 also decreased susceptibility of the cell line, Hep-2, to RSV [44]. However, since interferon expression was not altered in this cell line, the antiviral mechanism of this remains unknown [44].

1.5 HIV and LL-37

Sparse studies have suggested that LL-37 can inhibit HIV infection in cell lines and human primary cells [47–50]. Bergman and colleagues pre-incubated LL-37 with peripheral blood mononuclear cells (PBMC) and CD4+ T lymphocytes, the primary target of HIV, before addition of the virus. Using three different HIV isolates, they saw a significant dose-dependent inhibition of HIV infection in both cell types. However, when the virus itself was co-incubated with LL-37 before infecting PBMC, no inhibition of HIV infection was observed. This suggested that rather than acting on HIV itself, LL-37 demonstrates its anti-HIV activity by acting on the host cell or the host cell-virus interaction [47].

Wong and colleagues demonstrated that LL-37 can exhibit anti-HIV activity by inhibiting two fundamental enzymes: HIV-1 reverse transcriptase and HIV-1 protease. Recombinant HIV-1 reverse transcriptase was treated with LL-37 and assessed for the ability of the enzyme to

synthesize DNA from a template. LL-37 inhibited DNA synthesis by HIV-1 reverse transcriptase in a dose-dependent manner. Inhibition of recombinant HIV-1 protease by LL-37 was measured by the ability of the enzyme to cleave an artificial fluorogenic substrate. A decrease in fluorescence intensity compared to the untreated HIV-1 protease would thereby indicate enzyme inhibition. A slight inhibition (20-30%) was observed when HIV-1 protease was treated with LL-37. Remaining cognizant of the non-physiological, cell-free environment in which these experiments were conducted, LL-37 can nevertheless inhibit crucial HIV enzymes, underlining its anti-HIV activity (Figure 1)[50].

The anti-HIV activity of LL-37 was also investigated by Wang and colleagues to further determine the active fragments of the peptide. LL-37 and its truncated peptides (all chemically synthesized) were added to a 96-well plate. CEM-SS cells, a human T-lymphoblastoid cell line, which fuse with each other after HIV infection, were then added to the wells. [48]. Infection could therefore be quantified by the number of viral-induced syncytia formed between the cells [51]. Truncated peptides that were contained within the central helix of their parent LL-37 prevented HIV infection indicating the importance of this region of the AMP [48]. GF-17, which comprised the integral portion of the central helix, was even more efficacious in the inhibition of HIV than LL-37 [48]. These results clearly demonstrated anti-HIV properties of LL-37 and indicated the importance of the central helix on this anti-HIV activity. The comparative efficiency of its truncated peptides may also suggest that LL-37 contains sequences outside the central helix that counteract this desired activity.

The anti-HIV activity of LL-37 in other target cells have also been investigated, yielding somewhat conflicting results. Langerhans cells are a distinctive type of dendritic cells which reside in the mucosal epithelium [49,52]. As previously stated, Langerhans cells are often thought as

the first target of HIV infection [52]. Ogawa and colleagues isolated monocyte-derived Langerhans cells (mLCs) from monocyte-derived dendritic cells by flow cytometry and then treated the cells with LL-37. These cells were then infected with HIV and co-incubated with CD4+ T cells. HIV p24 antigen levels in the supernatant increased significantly after pre-treatment of mLCs with LL-37. Upon treatment of mLCs with LL-37, expression of HIV co-receptors, CD4 and CCR5, also increased thus making mLCs more vulnerable to HIV infection. As opposed to non-LC-like monocyte-derived dendritic cells (mDCs), which upon LL-37 treatment resulted in a decrease in HIV replication. This was potentially attributed to a decrease in CCR5 expression and/or a decrease in expression of DC-SIGN [49], an HIV receptor on dendritic cells [53]. Both Langerhans cells and dendritic cells are representative of initial HIV target cells in the vagina and are capable of transmitting the virus to T cells [3,53]. While the opposite results on the effects of LL-37 on HIV infection on mLCs versus mDCs are interesting, it is important to note that cells used in this study were monocyte-derived and may not be representative of cells in the genital epithelium. Transcriptomic analyses have highlighted the imperative role of the tissue environment in programming vaginal mucosal cell function [52,54,55].

The relationship between endogenous LL-37 expression and HIV infection has also been investigated. Levinson and colleagues measured LL-37 levels in cervicovaginal secretions (CVS) of high-risk HIV seronegative female sex workers in Kenya [56]. CVS that had the ability to neutralize HIV *in vitro* contained higher amounts of LL-37; however, higher levels of LL-37 were measured in CVS of participants who subsequently acquired HIV. It was nonetheless unlikely that the acquisition of HIV infection was caused by an increased amount of LL-37 in the CVS as these same individuals had higher rates of other sexually transmitted infections. Therefore, elevated levels of LL-37 was presumably a result of the presence of ongoing genital

infections and that LL-37 did not contribute to an increase in susceptibility to HIV [56]. Further studies by Levinson and colleagues went on to analyze innate immune factors in CVS of women from three groups: HIV-exposed seronegative; HIV positive; and low HIV risk [57]. Though there was a lack of observed differences in LL-37 levels among these three groups, those in the HIV-exposed seronegative group who had partners with a high plasma viral load showed higher levels of LL-37 and alpha-defensin HNP-1-3, suggesting HIV exposure elicited an immune response which includes the release of AMPs into genital secretions [57].

The mechanisms in which LL-37 enacts its anti-HIV effects have not been wholly investigated. As previously discussed, LL-37 can interfere with HIV reverse transcriptase and HIV protease [50]. LL-37 may also bind to anionic gangliosides such as GM3 of the infected host cells during HIV replication, thereby depleting the viral envelope of such lipids and rendering the virus less capable of infecting target cells [58,59]. LL-37 may directly damage the virus by binding to anionic lipids present on the HIV envelope, such as phosphatidylserine as well [60,61]. Similar to the central helix of LL-37, the V3 loop of the HIV-1 gp120 glycoprotein is cationic [62,63]. LL-37 may compete for the binding of the V3 loop to the HIV co-receptors (CCR5 and CXCR4) (Figure 1). LL-37 may also stimulate an increase of the Type I IFN response in target cells to mount a better immune response as in the study by He and colleagues [45]; though this has yet to have been tested in the context of HIV infection (Figure 1).

1.6 Immunomodulation via LL-37 may play a role in HIV infection

Conflicting results regarding the effect of LL-37 on HIV infection suggest the peptide has other roles that might counteract or enhance its anti-HIV activity such as the peptide's immunomodulatory properties. Aside from properties already discussed such as the upregulation

of CD4 and CCR5 in Langerhans cells [49] and upregulation of type I interferon signaling [45], LL-37 is known for a multitude of immunomodulatory properties. For example, LL-37 can upregulate the expression of chemokines and chemokine receptors, but can also downregulate inflammation by down-regulating inflammatory mediators such as C-C chemokine ligand 3 (CCL3), oncostatin M, IL-12, and TGF-β [64,65]. Chemotaxis of immune cells and stimulation of wound-healing can also be facilitated by LL-37 [64,66].

It is possible that LL-37 may play a part in increasing HIV infection because of its chemoattractant properties that lead to the recruitment and activation of monocytes and lymphocytes, the targets of HIV. LL-37 has been reported to both up-regulate and down-regulate the expression of anti-inflammatory and pro-inflammatory chemokines [66,67]. In terms of increasing infection, IL-8, a pro-inflammatory cytokine, can stimulate HIV replication in macrophages and T lymphocytes. Conversely and as mentioned above, LL-37 can increase production of IFN-α2, which can promote viral clearance [45]. Immunomodulation by LL-37 must therefore be considered if it is to be developed into an anti-HIV agent.

1.7 LL-37 as a possible Multipurpose Prevention Technology agent

Multipurpose Prevention Technology (MPT) refers to a single agent or combination of agents delivered in one device that can be used to protect individuals from both unwanted pregnancies and sexually transmitted infections (STIs) [68]. MPT development has been encouraged by The World Health Organization (WHO) as a response to unmet need and access to contraception for women alongside increasing rates of STIs (WHO 2020). While most MPTs in development involve the use of multiple products in a single delivery system, an LL-37-based

MPT will employ only one compound, namely LL-37 (or its truncated peptide forms), for dual contraceptive and anti-infective purposes.

Along with its anti-HIV activity, LL-37 is broadly bactericidal. This would be advantageous to its MPT development as LL-37 should also provide protection against other sexually transmitted pathogens [39] including *Neisseria gonorrhoeae,* which has shown a rise in antibiotic resistance [69,70]. LL-37 has also demonstrated microbicidal activity against *Treponema pallidum* (syphilis) and *Chlamydia trachomatis*[39]. LL-37 also has antiviral activity against the STI, herpes simplex virus I [71].

As for protection against unwanted pregnancies, LL-37 and its truncated peptides, including GF-17, uniquely exert spermicidal activity on both mouse (*in vitro* and *in vivo*) and human (*in vitro*) sperm [69,72]. The mechanism of spermicidal activity of these AMPs is analogous to that observed in bacteria. When these AMPs interact with anionic sulfogalactosylglycerolipid on the sperm head, this interaction results in sperm surface membrane disruption thus making the use of LL-37 (and/or its truncated peptides) as a vaginal contraceptive quite promising [72]. The wound-healing properties of LL-37 may furthermore aid in healing tears in the vaginal epithelium that may occur during intercourse, another potential benefit of this AMP [72].

Being an antimicrobial peptide naturally present in human bodily fluid, LL-37 is unlikely to be toxic when administered topically. Female mice that were vaginally administered LL-37 exhibited no histological damage to the reproductive tract nor induction of inflammatory cytokine production [72]. Mice also resumed fecundity once the peptide administration was halted[73]. LL-37 has displayed hemolytic ability, but concentrations needed for 50% lysis of human red blood cells were quite high (>100 μM) [74,75]. Few experiments have assesssed potential

cytotoxicity of LL-37 and its truncated peptides in human primary cells, but preliminary studies have indicated a lack of toxicity in HIV target cells [47,50]. The potential toxicity of LL-37 and its truncated peptides on the host cells, especially after challenge with HIV, as well as their immunomodulatory effects must be studied further. Safety of LL-37 has been demonstrated in clinical trials [76] and is currently being evaluated in several Phase II studies. Studies include the use of a formulated LL-37 cream for treatment of diabetic foot ulcers (ClinicalTrials.gov Identifier: NCT04098562) as well as intratumoral injections of LL-37 for treatment of melanoma (ClinicalTrials.gov Identifier: NCT02225366)[77,78]. Since ongoing studies demonstrate therapeutic efficacy and absence of toxicity, LL-37 has great potential for additional clinical applications warranting future clinical trials.

1.8 Modified and engineered LL-37, 17BIPHE2

LL-37 may represent a novel approach to HIV prevention as the AMP has reported ability to inhibit HIV infection in target cells including CD4+ T cells and dendritic cells [47,49]. Though contradictory data suggest LL-37 may also facilitate HIV infection as well as possess immunomodulatory properties that might counteract its inhibitory abilities [49,66]. The removal of such parts of the peptide reponsible for increasing HIV infection can be achieved by synthesizing truncated peptides. Shorter peptides will also be more economical to synthesize.

LL-37 and its truncated peptides still remain susceptible to degradation by numerous proteases, including those in cervicovaginal fluid; a drawback for vaginal administration [79]. Protease degradation can be circumvented by engineering modified peptides that contain one or more unnatural amino acids (e.g., D-amino acids), which cannot be recognized by human proteases and thus would evade degradation [80].

Wang and colleagues were aware of the potential efficacy of LL-37 derived peptides as microbicidal agents, particularly its truncated peptide, GF-17. In order to minimize protease degradation, they modified GF-17 into several engineered peptides and tested whether these newly synthesized peptides were capable of inhibiting the growth of multidrug resistant pathogens[81]. One such synthesized peptide, 17BIPHE2, was synthesized to contain five unnatural residues (including three D-amino acids) which could withstand protease degradation [81] . 17BIPHE2 caused growth inhibition of multidrug resistant pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Acinetobacter baumanii* at a minimum inhibitory concentration of 3.1 μM (lower than the parallel microbicidal concentration of GF-17) indicating its potential therapeutic efficacy[81].

Limited work has been undertaken to ensure the absence of potential toxicity of 17BIPHE2, though Wang and colleagues have denoted very minimal hemolytic ability of 17BIPHE2 and other GF-17 derivatives [81]. Preliminary work indicated that multiple administrations of 17BIPHE2 into the female mouse reproductive tract did not result in any histological injury to the vagina, cervix and uterus [73,79]. Similarly to LL-37, the female mice which received 17BIPHE2 treatment resumed full fecundity upon termination of the peptide administration [73,79]. Though 17BIPHE2 is a promising peptide, the anti-HIV activity of 17BIPHE2 and its effect on target cells has yet to be confirmed.

1.9 Research hypothesis and objectives

The anti-HIV activity of LL-37, and more notably 17BIPHE2, must be further investigated. It is hypothesized that LL-37 and/or 17BIPHE2 will inhibit HIV in immunologically relevant models of infection, including cell lines and primary cells.

In order to address whether these peptides possess anti-HIV activity, there are three main objectives:

1) Determine whether LL-37 and 17BIPHE2 can reduce HIV replication in chronically infected cells.

2) Determine whether LL-37 or 17BIPHE2 act directly on HIV, making it less able to infect target cells, and

3) Determine if LL-37 or 17BIPHE2 exert their anti-HIV activity by inducing changes to the target cells that make them less susceptible to infection.

Chapter 2: Materials and Methods

2.1 Reagents

2.1.1 Media

Gibco® Roswell Park Memorial Institute 1640 medium (RPMI-1640) with and without phenol red indicator, Gibco® Dulbecco's Modified Eagle's medium (DMEM), and Gibco® phosphate buffered saline (PBS), pH 7.4 were purchased from ThermoFisher Scientific (Ottawa, ON). Media was supplemented with heat-inactivated fetal bovine serum (FBS), penicillin (100units(U)/mL) and streptomycin (10 µg/mL) (PenStrep), and L-glutamine (ThermoFisher Scientific).

2.1.2 Virus Strains

HIV_{CS204} was obtained as a clinical isolate and able to use both CCR5 and CXCR4 as co-receptors (a gift from Dr. Francisco Diaz-Mitoma at the Children's Hospital of Eastern Ontario, Ottawa, ON, Canada). $HIV_{NL4.3}$ (ARP-2852, contributed by Dr. M Martin), a CXCR4-tropic virus, was obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH (Manassas, Virginia, USA).

2.1.3 Antimicrobial Peptides

LL-37 and 17BIPHE2 were chemically synthesized with >95% purity determined by high-performance liquid chromatography. LL-37 was synthesized by CPC Scientific (San Jose, CA, USA), whereas 17BIPHE2 was synthesized by Genemed Synthesis Inc. (San Antonio, TX, USA).

2.2 Cell Culture

2.2.1 Cell Lines

ACH-2 (ARP-349, contributed by Dr. Thomas Folks) cells were obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH. TZM-bl cells (ARP-8129, contributed by Dr. John C. Kappes and Dr. Xiaoyun Wu) were also obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH.

ACH-2 are a chronically infected T-lymphocyte clone containing one copy of integrated HIV-1 and can be stimulated to induce HIV-1 replication using 12-phorbol myristate 13-acetate (PMA) at 10 ng/ml [82,83]. ACH-2 cells were cultured in suspension in RPMI-1640 medium, supplemented with 10% FBS, PenStrep, and L-Glutamine (2 mM), in T75 flasks. Cells were incubated at $0.2x10^5$ to $1x10^6$cells/ml by passaging every 2-3 days in a volume of 15ml.

TZM-bl are a reporter cell line that express luciferase under the HIV-1 promoter[84]. TZM-bl (6×10^6 cells) were cultured in DMEM with 10% FBS and PenStrep and L-glutamine (2 mM) in T75 cell culture flasks (Falcon™, Fisher Scientific), and routinely subcultured every 2-3 days to maintain the cell monolayer. At approximately 80-85% confluency, cells were detached using TrypLE (Gibco™, ThermoFisher Scientific) and re-plated at a 1:5 dilution resulting in a concentration of $0.1\text{-}0.2x10^6$ cells/ml, in a total volume of 15 ml per T75 flask. All cells were cultured at 5% CO_2, 37°C.

2.2.2 Isolation and activation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was drawn from healthy donors into sterile 60 ml syringes, containing 100U/ml filter-sterilized Heparin Sodium (LEO Pharma Inc., Thornhill, ON). Following

collection, 30 ml of whole blood was layered over 15 ml of Lymphoprep™ density gradient medium (Stemcell Technologies, Vancouver, BC) and centrifuged at 400 x g for 30 minutes (Megafuge 1.0, Heraeus Instruments, Germany) without braking. The buffy coats from individual donors were then collected into 50 ml Falcon tubes and topped up with PBS to 50 ml. Cells were pelleted by centrifugation (300 x g for 20 min). Cell pellets from individual donors were then pooled in one 50ml Falcon tube, and cells were washed twice with PBS (400 x g for 10min). Following the final wash, peripheral blood mononuclear cells (PBMC) were counted by trypan blue exclusion and resuspended at $2x10^6$ cells/ml in RPMI-1640 medium, supplemented with 10% FBS, PenStrep, and L-Glutamine (2 mM). Cells were activated with 30U/ml IL-2 (Cell Sciences, Canton, MA) and 5 µg/ml phytohaemagglutinin (PHA) (Sigma-Aldrich, Oakville, ON, Canada) for 3 days at 5% CO_2, 37°C. Healthy blood donors provided written informed consent to partake in the study. This was approved by The Ottawa Health Science Network Research Ethics Board.

2.2.3 $CD4^+$ T cell isolation and activation

PBMC were resuspended at $5x10^7$ cells/ml in the manufacturer's recommended sorting buffer (PBS, 0.5% FBS, and 2mM ethylenediaminetetraacetic acid (EDTA, pH 8; Sigma Aldrich). CD4+ T cells were then isolated from PBMC by negative selection using the EasySep™ Human CD4+ T cell Enrichment Kit and EasySep™ Magnet (StemCell Technologies, Vancouver, BC), in accordance with the manufacturer's protocol. The negative fraction was then counted and resuspended at $2x10^6$ cells/ml in RPMI-1640 medium supplemented with 10% FBS, PenStrep, and L-glutamine (2mM). Purity was routinely assessed by flow cytometry immediately post-sort using a CD4 antibody (clone SK3; BioLegend, San Diego, CA). Isolated CD4+ T cells were activated with 30 U/ml IL-2 (Cell Sciences, Canton, MA) and 5µg/ml PHA (Sigma-Aldrich) for three days at 5% CO_2, 37°C.

2.3 Virus amplification using CD8-depleted PBMCs

Virus strains were amplified using activated CD8-depleted healthy donor PBMC. CD8+ cells were removed using EasySep™ Human CD8 Positive Selection Kit II and EasySep™ Magnet (StemCell Technologies). Cells were activated using 30U/ml IL-2 (Cell Sciences, Canton, MA) and 5 µg/ml PHA (Sigma-Aldrich, Oakville, ON, Canada) for 3 days at 5% CO_2, 37°C in T75 cell culture flasks (Falcon™, Fisher Scientific). Activated cells were counted and divided in to three 50 ml Falcon tubes. Cells were then administered 1 ml of medium (Mock), $HIV_{NL4.3}$, or HIV_{CS204} and incubated for 2 h at 37°C. $2x10^7$ cells from each tube were then resuspended at $2x10^6$ cells per ml of medium with 30U/ml IL-2 and placed into three separate flasks. The following day, fresh activated CD8-depleted PBMCs were evenly distributed to each flask at $2x10^6$ cells per ml. This was repeated an additional three times. The virus stocks and the mock cultures were then harvested by aspirating the cells and supernatant from each flask into 50 ml Falcon tubes. Cells were pelleted by centrifugation at 400 x g for 10 min and the supernatants were collected. The supernatants were then filtered first with a 0.45 micron filter followed by filtration with a 0.2 micron filter. The cell-free virus stocks were aliquoted at 1 ml per vial and stored frozen at -80°C. HIV-1 concentration of viral stocks was quantified via Enzyme-linked immunosorbent assay (ELISA) for p24 antigen (p24) (See section 2.4.1).

2.4 Infection and Quantification of Infection

2.4.1 Enzyme-linked immunosorbent assay (ELISA) for p24 antigen (p24)

ELISA was used to determine the capsid protein concentration of HIV-1 (p24) from cell cultures infected with HIV to quantify infection and viral replication. This assay and its reagents were provided by the AIDS and Cancer Virus Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research (Frederick, MD, USA), supported with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. Supernatants of the cell cultures were incubated for 1 hour at 37°C with 1% Triton X-100. This incubation is necessary for the lysis of any remaining cells and inactivation of HIV. The ELISA assay was then performed according to the manufacturer's protocol. Samples were run on each plate in duplicate. Absorbance was read at 450nm wavelength with a reference wavelength of 650 nm using the Multiskan Ascent 96 Plate Reader (ThermoFisher Scientific).

2.4.2 Effect of LL-37 and 17BIPHE2 on chronically infected cell line, ACH-2

ACH-2 cells ($5x10^5$ cells/tube, $1x10^6$ cells/ml) were simultaneously stimulated with 10ng/ml phorbal 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and treated with LL-37 or 17BIPHE2 (0, 2.8, 5.6, or 11.2 μM) for 2 h, then washed with PBS (470 x g for 5 min). Concentrations of peptides used were based on a molar conversion of the concentrations used by Bergman et al. (2007) [47]. Cells were further cultured in RPMI-1640 medium for 3 days. Cells were then centrifuged (470 x g for 5 min) and supernatants were collected and any remaining cells were lysed with 1% Triton X-100 for p24 ELISA (See Figure 4).

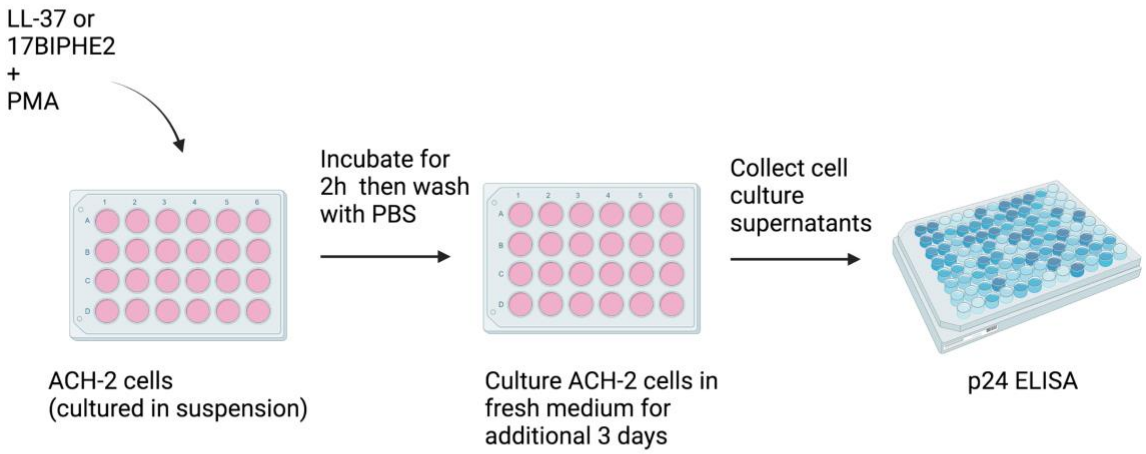

LL-37 or
17BIPHE2
+
PMA
Incubate for
2h then wash
with PBS
Collect cell
culture
supernatants
ACH-2 cells
(cultured in suspension)
Culture ACH-2 cells in
fresh medium for
additional 3 days
p24 ELISA

Figure 4. Effect of LL-37 and 17BIPHE2 on chronically infected cell line, ACH-2

Schematic representation depicting the treatment of ACH-2 cells with LL-37 or 17BIPHE2.

Image created with BioRender.com

2.4.3 Effects of 17BIPHE2 on the HIV_{CS204} infection of the TZM-bl reporter cell line

In the first set of experiments, TZM-bl cells were infected with HIV_{CS204} (25 ng p24/ml), which were pre-incubated with 17BIPHE2 (0, 5.6, 11.2, or 22.4 μM) for 2 h. The infection period was 2 h (Figure 5). In the second set of experiments, both HIV_{CS204} (25 ng p24/ml) and 17BIPHE2 (0, 5.6, 11.2, or 22.4 μM) were simultaneously added to the TZM-bl cell cultures and the infection period was likewise kept for 2 h (Figure 6). Note that 17BIPHE2 was present during the infection period in both sets of experiments. All infection experiments also included a Mock condition. When viral stocks were made by amplifying the virus on PBMC and collecting the supernatant, a Mock stock was made in parallel which consisted of PBMC supernatant that were not infected with the virus. After infection, cells were washed with PBS and fresh medium was added. Cells were cultured for 2 days before lysis with Glo-lysis buffer (Promega) and performance of luciferase assay (Bright-Glo™ Luciferase Assay System, Promega). Luciferase was read using a BMG LumiStar Optima Microplate Reader (BMG LabTech, Allmendgrün, Ortenberg, Germany).

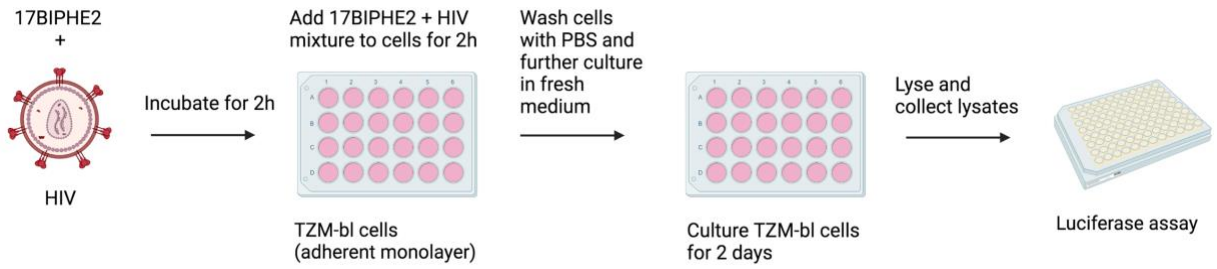

17BIPHE2
+
HIV
Incubate for 2h
Add 17BIPHE2 + HIV mixture to cells for 2h
TZM-bl cells (adherent monolayer)
Wash cells with PBS and further culture in fresh medium
Culture TZM-bl cells for 2 days
Lyse and collect lysates
Luciferase assay

Figure 5. Infection of TZM-bl cells with HIV pre-treated with 17BIPHE2

Schematic representation depicting the infection of TZM-bl cells with HIV pre-treated with 17BIPHE2. Image created with BioRender.com

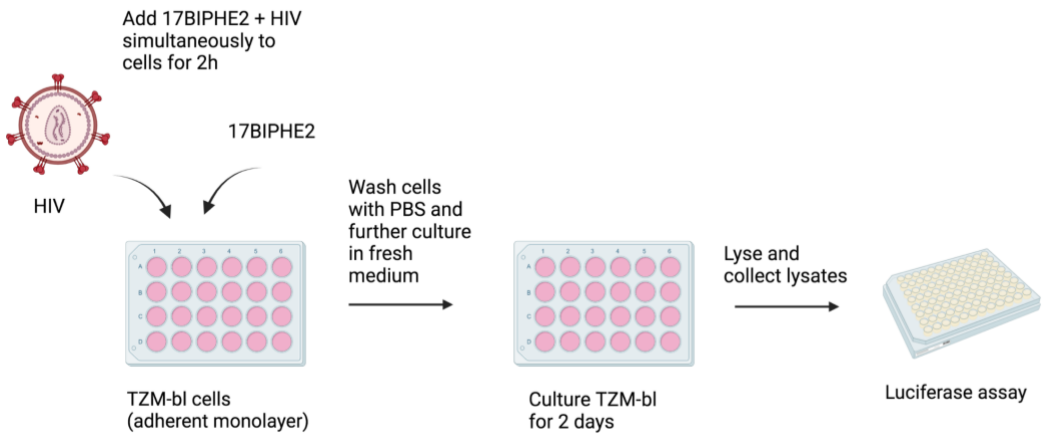

Add 17BIPHE2 + HIV
simultaneously to
cells for 2h
17BIPHE2
HIV
Wash cells
with PBS and
further culture
in fresh
medium
Lyse and
collect lysates
TZM-bl cells
(adherent monolayer)
Culture TZM-bl
for 2 days
Luciferase assay

Figure 6. Simultaneous addition of HIV and 17BIPHE2 to TZM-bl cells

Schematic representation depicting the simultaneous HIV infection and 17BIPHE2 treatment of TZM-bl cells. Image created with BioRender.com

2.4.4 Infection of activated PBMC or isolated CD4+ T cells with HIV pre-treated with 17BIPHE2

HIV_{CS204} (150 ng p24/ml) or $HIV_{NL4.3}$ (200 ng p24/ml), used for infecting activated PBMC or CD4+ T cells respectively, was incubated with 17BIPHE2 (0, 5.6, 11.2, or 22.4 μM) for 2 h before adding to activated cells. The infection period was for 2 h. Cells were then washed with PBS (470 x g for 5 min) then cultured in medium with 30 U/ml IL-2 for 4-5 days. Cells were then centrifuged (470 x g for 5 min) and supernatants were collected and any remaining cells were lysed with 1% Triton X-100 for p24 ELISA (See Figure 7).

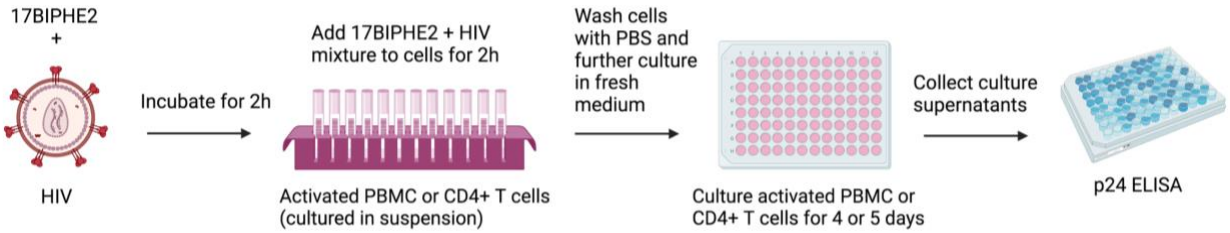

17BIPHE2
+
HIV
Incubate for 2h
Add 17BIPHE2 + HIV
mixture to cells for 2h
Activated PBMC or CD4+ T cells
(cultured in suspension)
Wash cells
with PBS and
further culture
in fresh
medium
Culture activated PBMC or
CD4+ T cells for 4 or 5 days
Collect culture
supernatants
p24 ELISA

Figure 7. Infection of activated PBMC or isolated CD4+ T cells with HIV pre-treated with 17BIPHE2

Schematic representation depicting the infection of activated PBMC or CD4+ T cells with HIV pre-treated with 17BIPHE2. Image created with BioRender.com

2.4.5 Pre-incubating 17BIPHE2 with activated PBMC or isolated CD4+ T cells prior to HIV infection

Activated PBMC or CD4+ T cells were incubated with 17BIPHE2 (0, 5.6, 11.2, or 22.4 μM) for 2 h. After 2 h, cells were washed with PBS (400 x g for 5min), then infected with HIV_{CS204} (150 ng p24/ml) or $HIV_{NL4.3}$ (200 ng p24/ml) for 2 h. Cells were washed with PBS (400 x g for 5 min), resuspended in fresh medium with 30 U/ml IL-2 and incubated for 4-5 days. Cells were then centrifuged (470 x g for 5 min) and supernatants were collected and any remaining cells were lysed with 1% Triton X-100 for p24 ELISA (See Figure 8).

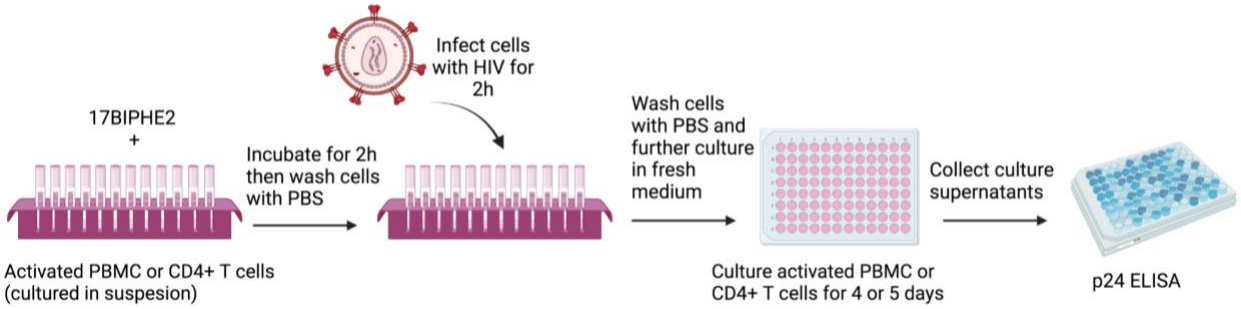

17BIPHE2
+
Activated PBMC or CD4+ T cells
(cultured in suspesion)
Incubate for 2h
then wash cells
with PBS
Infect cells
with HIV for
2h
Wash cells
with PBS and
further culture
in fresh
medium
Culture activated PBMC or
CD4+ T cells for 4 or 5 days
Collect culture
supernatants
p24 ELISA

Figure 8. Pre-incubating 17BIPHE2 with activated PBMC or isolated CD4+ T cells prior to HIV infection

Schematic representation depicting the pre-treatment of activated PBMC or CD4+ T cells with 17BIPHE2 prior to HIV infection. Image created with BioRender.com

2.5 Investigating cell surface markers on CD4+ T cells

CD4+ T cells (2x 10^5 cells per tube, 100 µl) were incubated with 17BIPHE2 (0, 5.6, 11.2, or 22.4 µM) for 2 h in 5 ml polypropylene tubes (Falcon™, Fisher Scientific), washed with PBS (400 x g for 5min), then re-suspended in 300 µl PBS and incubated with fluorochrome labeled antibodies against cell surface markers CD69, HLA-DR, CXCR4, and CCR5 (See Table 1). Cells were washed again prior to flow cytometric analysis. Flow cytometric analysis was performed using the CytoFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA) with the CytExpert Software (Beckman Coulter) and the results were further analyzed using FlowJo Software (BD Biosciences, Mississauga, ON, Canada).

Table 1. Monoclonal antibodies used in flow cytometry

Target	Reactivity	Species	Product Number	Company	Clone	Conjugated fluorophore	Volume per test
CD4	Human	Mouse	340443	BD Biosciences	SK3	APC	5 µl
CCR5	Human	Rat	359106	BioLegend	J418F1	PE	5 µl
CXCR4	Human, African Green, Baboon, Chimpanzee, Cynomolgus, Rhesus, Sooty Mangabey	Mouse	306528	BioLegend	12G5	APC/Cyanine7	5 µl
HLA-DR	Human, Cross-Reactivity: Rhesus	Mouse	327022	BioLegend	LN3	APC	5 µl
CD69	Human	Mouse	347823	BD Biosciences	L78	FITC	10 µl

2.6 Cell Viability Assays

2.6.1 Calcein-AM and propidium iodide

Invitrogen Calcein-AM (ThermoFisher Scientific), a cell-permeant dye that is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases in live cells was used to determine cell viability in both TZM-bl and activated isolated CD4+ T cells that

were incubated with 17BIPHE2 for 2 h. Propidium Iodide (PI) (Biolegend, San Diego, CA, USA) was used to determine cell death.

Cells (2x 10^5 cells per tube, 100 µl) were incubated for 2 h in 5 ml polypropylene tubes with 17BIPHE2 (0, 5.6, 11.2, or 22.4 µM) or 500 µM carbonyl cyanide m-chlorophenyl hydrazine (CCCP), which disrupts ATP synthesis during oxidative phosphorylation, as the positive control. Cells were then washed with PBS (400 x g for 5 min), then re-suspended in 1ml PBS and added with Calcein-AM following the manufacturer's provided protocol. Cells with green-fluorescent calcein were analyzed for both numbers and fluorescence intensity by flow cytometry (Ex. 488-nm, Em. 525/40-nm) using the CytoFLEX Flow Cytometer (Beckman Coulter) and CytExpert Software (Beckman Coulter) and the results were further analyzed using FlowJo Software (BD Biosciences).

In parallel, cells (2x 10^5 cells per tube, 100 µl) were incubated with 17BIPHE2 (0, 5.6, 11.2, or 22.4 µM) or 0.1% Saponin, a detergent which causes cell death, as the positive control for 2 h in 5 ml polypropylene tubes (Falcon™, Fisher Scientific), washed with PBS (400 x g for 5min), then re-suspended in 300 µl PBS and stained with PI (1 µg/ml). Cells stained with PI were analyzed for both numbers and fluorescence intensity by flow cytometry (Ex. 488-nm, Em. 610/20-nm) using the CytoFLEX Flow Cytometer (Beckman Coulter) with the CytExpert Software (Beckman Coulter) and the results were further analyzed using FlowJo Software (BD Biosciences).

2.6.2 Annexin V- DY-634 PI Apoptosis Staining

Annexin V-DY-634 PI Apoptosis Staining/Detection Kit (abcam, Cambridge, UK) was used to determine apoptosis in ACH-2 as the kit allows cells to be fixed with paraformaldehyde (PFA). ACH-2 (2x 10^5 cells per tube, 100 µl) were incubated with 6 µM camptothecin, a

topoisomerase inhibitor, (Sigma Aldrich) as the positive control for Annexin V for 24 h, as optimized in our laboratory. On the following day, ACH-2 (2x 10^5 cells per tube, 100 µl) were incubated with 17BIPHE2 (0, 5.6, 11.2, or 22.4 µM) or 0.1% Saponin as the positive control for PI for 2 h in 5 ml polypropylene tubes (Falcon™, Fisher Scientific) then washed. Cells were stained in accordance with manufacturer's protocol and fixed with 2% PFA for 20 min. Cells stained with Annexin V-DY-634 (Ex. 638-nm, Em. 660/20-nm) and PI (Ex. 488-nm, Em. 610/20-nm) were assessed concurrently for numbers and fluorescence intensity using the CytoFLEX Flow Cytometer (Beckman Coulter) and CytExpert Software (Beckman Coulter) and further analyzed using FlowJo Software (BD Biosciences).

2.6.3 JC-1

Molecular Probes MitoProbe™ JC-1 (5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) Assay Kit for Flow Cytometry (ThermoFisher Scientific) was also used to confirm CD4+ T cell viability in accordance with the manufacturer's protocol. CCCP was again used as the positive control at 200 µM. The JC-1 dye accumulation in mitochondria is dependent on the mitochondrial membrane potential, the status of which could be revealed by the fluorescence emission shift between the red (~590 nm) and green (~529 nm) fluorescence. In cells with healthy mitochondria, JC-1 will aggregate and fluoresce red (~590 nm) whereas in cells with compromised mitochondria, JC-1 remains in its monomeric form and fluoresces green (~529 nm). Mitochondrial depolarization is thus indicated by a lower red/green fluorescence ratio. This was measured by flow cytometry using the instrumentation stated above and compensated using FlowJo Software.

2.7 Statistics

Statistics were performed using GraphPad Prism 8 software (San Diego, CA). One-way ANOVA was used and P values ≤ 0.05 were considered significant.

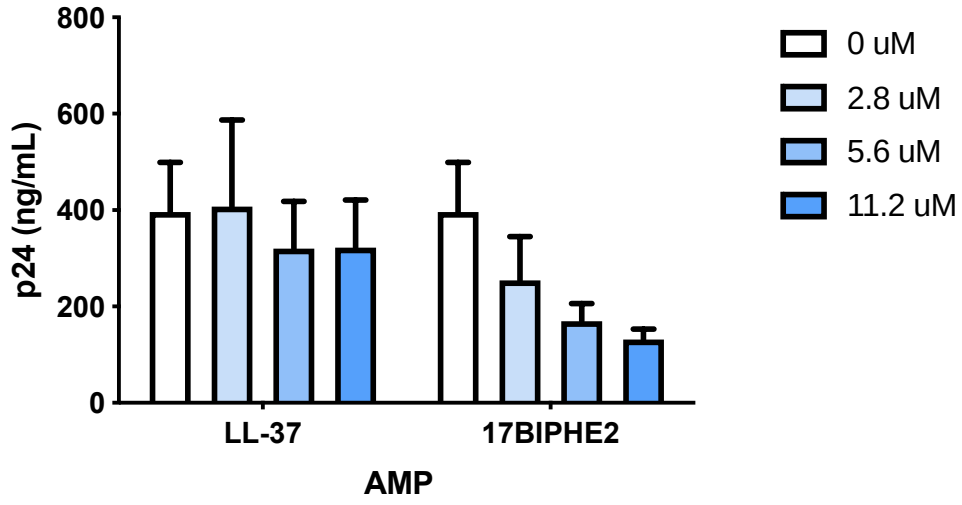
Chapter 3: Results

3.1 17BIPHE2, but not LL-37, reduces HIV replication in chronically infected cells

The chronically infected T-lymphocyte clone, ACH-2, can be stimulated to induce productive HIV-1 using PMA at 10 ng/ml [82,83]. These cells were used to determine whether LL-37 or 17BIPHE2 could decrease HIV replication in a chronically infected cell line.

ACH-2 cells were stimulated with PMA and administered either LL-37 or 17BIPHE2 at 2.8, 5.6, and 11.2 µM for 2 h, followed by a wash and subsequent culture for 3 days prior to supernatant collection for p24 ELISA. 17BIPHE2 significantly (One-way ANOVA, $p \leq 0.05$) decreased HIV p24 production in a dose-dependent manner which was measured by ELISA (Figure 9), whereas LL-37 did not exhibit the same trend. Since LL-37 was not as successful at reducing HIV replication in this model, 17BIPHE2 was chosen to proceed with subsequent experiments.

A


800
600
400
200
0
p24 (ng/mL)
LL-37
17BIPHE2
AMP
0 uM
2.8 uM
5.6 uM
11.2 uM

B

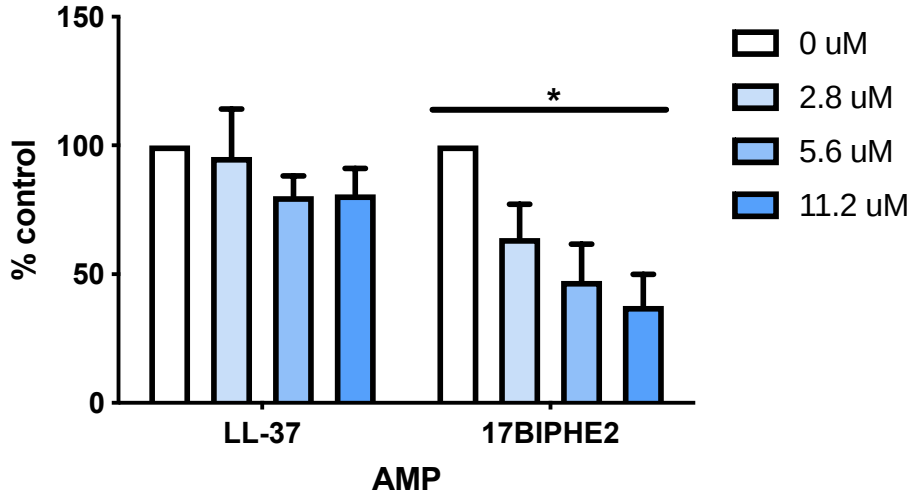

150
100
50
0
% control
*
LL-37
17BIPHE2
AMP
0 uM
2.8 uM
5.6 uM
11.2 uM

Figure 9. 17BIPHE2, but not LL-37, reduces HIV replication in chronically infected cells

ACH-2 cells were simultaneously stimulated with PMA and treated with LL-37 or 17BIPHE2 for 2 h, then washed. Cells were further incubated for 3 days. Supernatants were collected for p24 ELISA. A) Raw data depicting p24 concentration (ng/ml). B) Normalized data to untreated group (0 µM AMP). *n=3, one-way ANOVA,* * $p \leq 0.05$

3.2 Investigating the ability of 17BIPHE2 to reduce HIV infection by acting directly on HIV, making it less able to infect target cells

3.2.1 Pre-treatment of HIV with 17BIPHE2 before infecting TZM-bl, a reporter cell line

The ability of 17BIPHE2 to act on the virus to make it less infective was first investigated using the TZM-bl reporter cell line. The dual tropic strain, HIV_{C2S04} (25ng p24/ml), was pre-incubated with 17BIPHE2 for 2 h before the virus and peptide mixture was added to TZM-bl cells for 2 h. TZM-bl are a useful cell line to study HIV infection as they express luciferase under the HIV-1 promoter, thus infection can be quantified by light production [84]. After infection, cells were washed with PBS and fresh medium was added. After 48 h, TZM-bl cells were again washed then lysed with Glo-lysis buffer. Lysates were used for the luciferase assay to measure luminescence. A significant (One-way ANOVA, $p \leq 0.01$) decrease in luminescence occurred in a dose-dependent manner after virus incubation with 17BIPHE2 prior to infection, indicating a decrease in HIV infection (Figure 10).

3.2.2 Pre-treatment of HIV with 17BIPHE2 before infecting activated PBMC

PBMC were then used as a more physiologically relevant model as they encompass multiple HIV target cells. PBMC were isolated from blood from healthy donors then stimulated with IL-2 (30 U/ml) and PHA (5 µg/ml) for three days in order for them to be activated and more susceptible to HIV infection. HIV_{C2S04} (150 ng p24/ml) was incubated with 17BIPHE2 for 2 h before the mixture was added to activated cells for 2 h, paralleling the prior TZM-bl experiment. Cells were cultured for 4 days or 5 days before supernatant collection. The supernatants were then assessed for p24 antigen concentration via ELISA as a quantification of HIV infection.

Contrasting with the previous experiment, 17BIPHE2 incubation with HIV prior to the addition to PBMC did not significantly alter p24 production after 4 days nor 5 days (Figure 11).

3.2.3 Pre-treatment of HIV with 17BIPHE2 before infecting activated isolated CD4+ T cells

Though 17BIPHE2 was not effective in decreasing infection in activated PBMC when pre-incubated with HIV, it remained unclear whether this pre-incubation could have an impact on the infection of a more specific target, CD4+ T cells. CD4+ T cells were isolated from PBMC and activated with IL-2 and PHA. The X4-tropic virus $HIV_{NL4.3}$ (200 ng p24/ml) was used as activated peripheral blood CD4 lymphocytes express mostly CXCR4, rather than CCR5 as their HIV co-receptor [85,86]. Nonetheless, 17BIPHE2 incubation with HIV prior to the mixture addition to CD4+ T cells did not alter p24 production; a similar result to the previous experiment in PBMC (Figure 12).

3.2.4 Simultaneously adding HIV and 17BIPHE2 to TZM-bl

Since the primary cell experiments did not corroborate the results from pre-incubating HIV with 17BIPHE2 prior to infecting TZM-bl, the effect of simultaneously adding HIV and 17BIPHE2 to TZM-bl was examined to try to determine if the initial 2 h pre-incubation was an important component of the observed activity of 17BIPHE2. HIV_{CS204} (25 ng p24/ml) and 17BIPHE2 were thus simultaneously added to TZM-bl cells and incubated for 2 h after which cells were washed and fresh medium was added. Cells were then cultured for 2 days before lysis and performance of luciferase assay. If simultaneous addition of HIV and 17BIPHE2 exhibited similar results as pre-incubating the virus with 17BIPHE2, it may be that 17BIPHE2 does not act directly on the virus itself, but rather on the cell or virus/cell interaction. The results indicated a significant (One-way ANOVA, $p \leq 0.05$) dose-dependent decrease in luminescence, similar to

that of the experiments in which virus and peptide were pre-incubated prior to infection (Figure 13).

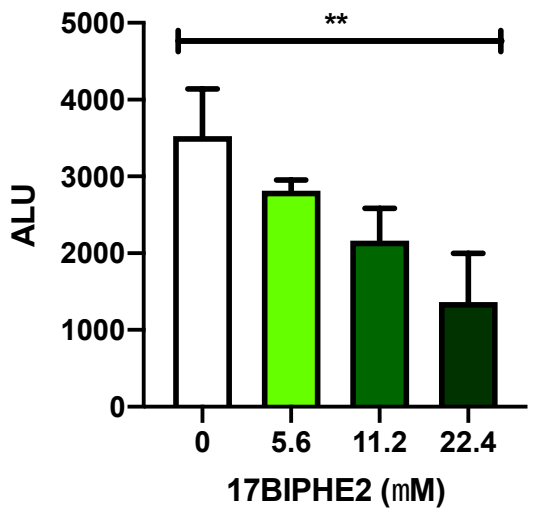

**
5000
4000
3000
2000
1000
0
ALU
0
5.6
11.2
22.4
17BIPHE2 (mM)

Figure 10. Effect of pre-incubating HIV with 17BIPHE2 before infecting TZM-bl.

HIV_{CS204} (25 ng p24/ml) was pre-incubated with 17BIPHE2 for 2 h before addition to TZM-bl cells for 2 h. After infection, cells were washed and fresh medium was added. Cells were cultured for 2 days before lysis and luciferase assay with measurement of arbitrary luminescence units (ALU). Values for the mock HIV treatment group were subtracted from each ALU value. n=3, One-way ANOVA, ** $p \leq 0.01$

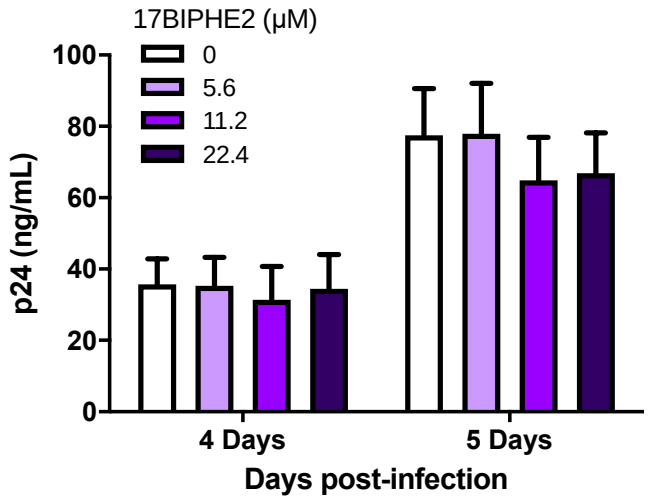

17BIPHE2 (µM)
0
5.6
11.2
22.4
100
80
60
40
20
0
p24 (ng/mL)
4 Days
5 Days
Days post-infection

Figure 11. Effect of pre-incubating HIV with 17BIPHE2 before infecting activated PBMC. HIV_{CS204} (150 ng p24/ml) was incubated with 17BIPHE2 for 2 h before being added to activated PBMC for 2 h after which cells were washed and cultured in fresh medium for 4-5 days before supernatant collection and subsequent p24 ELISA. n=3, One-way ANOVA, ns.

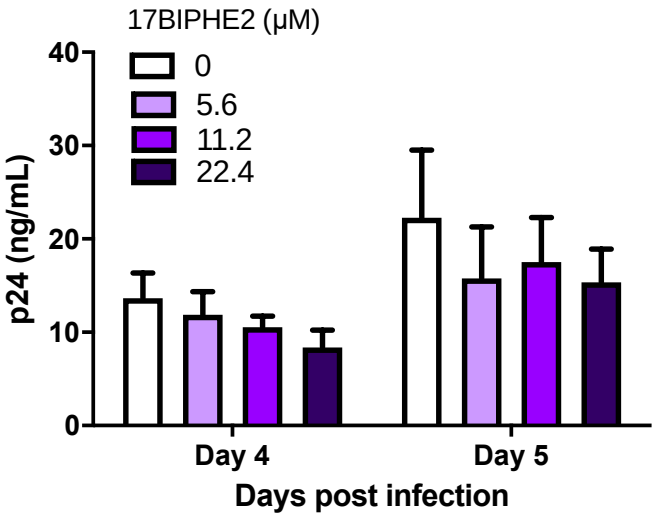

17BIPHE2 (μM)
0
5.6
11.2
22.4
40
30
20
10
0
p24 (ng/mL)
Day 4
Day 5
Days post infection

Figure 12. Effect of pre-incubating HIV with 17BIPHE2 before infecting activated isolated CD4+ T cells.

$HIV_{NL4.3}$ (200 ng p24/ml) was incubated with 17BIPHE2 for 2 h before being added to activated CD4+ T cells for 2 h after which cells were washed and cultured for 4-5 days before supernatant collection and subsequent p24 ELISA. n=4, One-way ANOVA, ns.

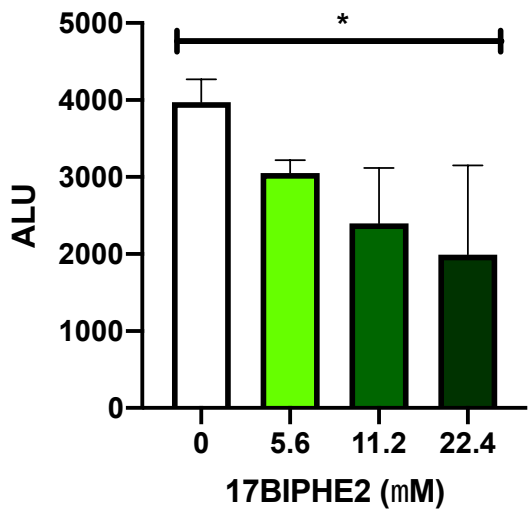

*
5000
4000
3000
2000
1000
0
ALU
0
5.6
11.2
22.4
17BIPHE2 (mM)

Figure 13. Effect of simultaneously adding HIV and 17BIPHE2 to TZM-bl cells.

HIV_{CS204} (25 ng p24/ml) and 17BIPHE2 were simultaneously added to TZM-bl cells for 2 h after which cells were washed and fresh medium was added. Cells were cultured for 2 days before lysis and performance of luciferase assay with measurement of arbitrary luminescence units (ALU). Values for the mock HIV treatment group were subtracted from each ALU value. n=3, One-way ANOVA, * $p \leq 0.05$

3.3 Investigating the ability of 17BIPHE2 to reduce HIV infection by acting directly on the target cells

3.3.1 Pre-incubating PBMC with 17BIPHE2 before HIV infection

To explore whether 17BIPHE2 can decrease HIV infection by altering target cells or interfering with the virus/cell interaction, cells were incubated with 17BIPHE2 prior to HIV infection. Activated PBMC were incubated with 0, 5.6, 11.2, or 22.4 μM 17BIPHE2 for 2 h and then washed. After 2 h, cells were infected with HIV_{CS204} (150 ng p24/ml) for 2 h. Cells were then washed and incubated for 4-5 days. After 4-5 days, supernatants were collected for subsequent p24 ELISA. Incubation of PBMC with 17BIPHE2 caused a significant (One-way ANOVA, * $p \leq 0.001$, ** $p \leq 0.0001$) decrease in p24 production in a dose-dependent manner both after 4 days and 5 days, with a mean decrease in infection of about 67% and 79% respectively at the highest concentration (Figure 14).

3.3.2 Pre-incubating CD4+ T cells with 17BIPHE2 before HIV infection

It was then investigated whether this observed result of a decrease in p24 production could be recapitulated in isolated activated CD4+ T cells. Similar to the previous experiment, activated CD4+ T cells were incubated with 0, 5.6, 11.2, or 22.4 μM 17BIPHE2 for 2 h and then washed. After 2 h, cells were infected with $HIV_{NL4.3}$ (200 ng p24/ml) for 2 h. Cells were also washed and incubated for 4-5 days. After 4-5 days, supernatants were collected for subsequent p24 ELISA. When CD4+ T cells were pre-incubated with 17BIPHE2, a significant (One-way ANOVA, * $p \leq 0.05$) decrease in p24 production in a dose-dependent manner occurred after 4 days with a mean decrease in infection of about 50% at 22.4 μM 17BIPHE2 (Figure 15). There was a similar trend after 5 days, though it failed to reach statistical significance.

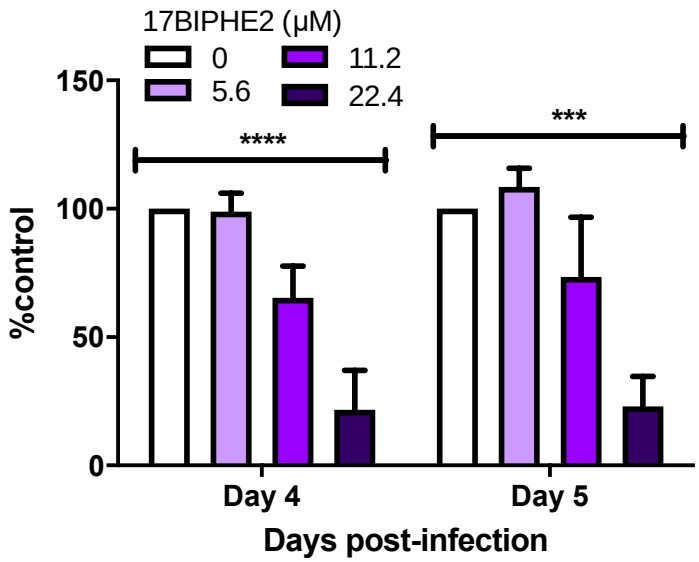

17BIPHE2 (µM)
0
5.6
11.2
22.4
150
100
50
0
%control
****
***
Day 4
Day 5
Days post-infection

Figure 14. Effect of pre-incubating activated PBMC with 17BIPHE2 before HIV infection. Activated PBMC were incubated with 17BIPHE2 for 2 h. After 2 h, cells were infected with HIV_{CS204} (150 ng p24/ml) for 2 h. Cells were washed and incubated for 4-5 days. Supernatant was collected for p24 ELISA. n=3, One-way ANOVA, *** $P \leq 0.001$, **** $P \leq 0.0001$.

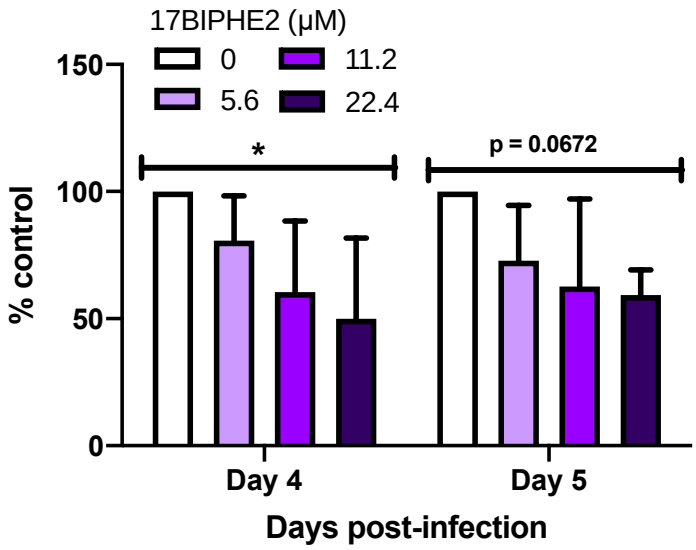

17BIPHE2 (μM)
0
11.2
5.6
22.4
150
100
50
0
% control
*
p = 0.0672
Day 4
Day 5
Days post-infection

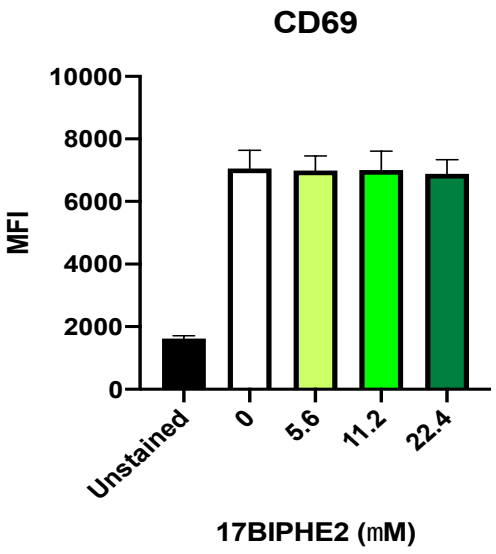
Figure 15. Effect of pre-incubating activated isolated CD4+ T cells with 17BIPHE2 before HIV infection.

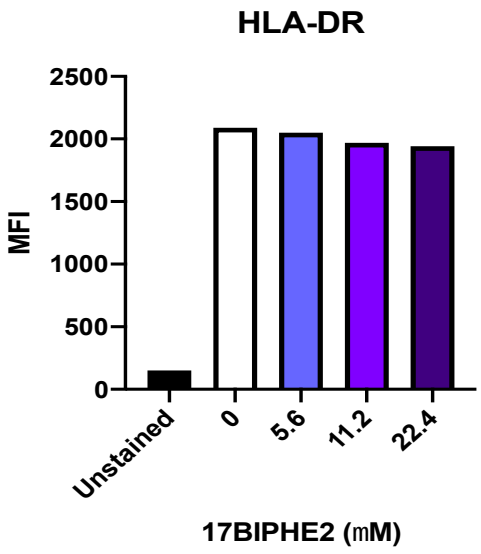
Activated isolated CD4+ T cells were incubated with 17BIPHE2 for 2 h. After 2 h, cells were infected with $HIV_{NL4.3}$ (200 ng p24/ml) for 2 h. Cells were washed and incubated for 4-5 days. Supernatant was collected for p24 ELISA. n=4, One-way ANOVA, * $P \leq 0.05$.

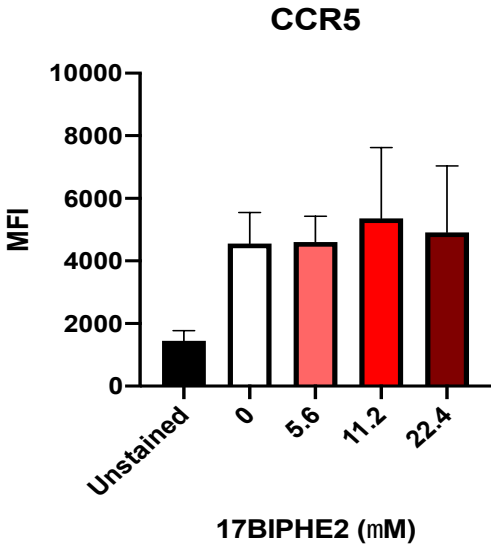
3.4 Potential mechanisms of HIV inhibition by 17BIPHE2

Possible mechanisms of HIV inhibition by 17BIPHE2 could be attributed to an induction of differential expression of cell surface markers [49]. Activation markers were assessed as activation of CD4+ T cells can facilitate HIV-1 infection. Hence, expression of lymphocyte activation markers CD69 and HLA-DR were assessed. Since LL-37 can influence expression of the HIV co-receptor, CCR5[49] , the effect of 17BIPHE2 on the expression of HIV co-receptors CXCR4 and CCR5 were also measured.

Isolated CD4+ T cells were incubated with 17BIPHE2 for 2 h, live cells were then washed then individually stained for cell surface markers. A list of antibodies used can be found in Table 1. Incubation with 17BIPHE2 did not change cell surface markers of activation nor co-receptor expression (Figure 16). This thus indicated that 17BIPHE2 acts to inhibit HIV through other mechanisms that have yet to be studied.


CD69
10000
8000
6000
4000
2000
0
MFI
Unstained
0
5.6
11.2
22.4
17BIPHE2 (mM)


HLA-DR
2500
2000
1500
1000
500
0
MFI
Unstained
0
5.6
11.2
22.4
17BIPHE2 (mM)


CCR5
10000
8000
6000
4000
2000
0
MFI
Unstained
0
5.6
11.2
22.4
17BIPHE2 (mM)

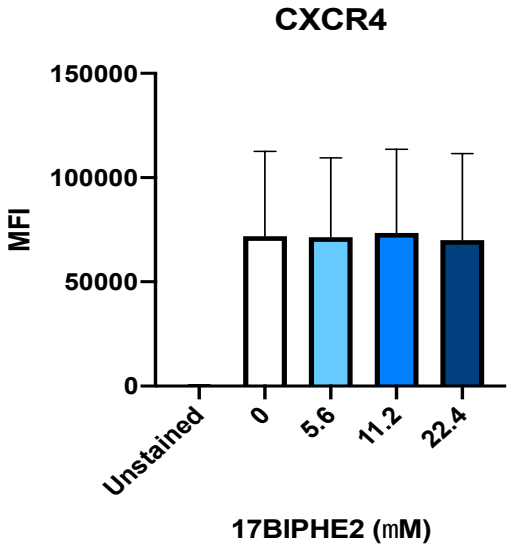

CXCR4
150000
100000
50000
0
MFI
Unstained
0
5.6
11.2
22.4
17BIPHE2 (mM)

Figure 16. Characterization of CD4+ T Cells following incubation with 17BIPHE2.

Activated isolated CD4+ T cells were incubated with 17BIPHE2 for 2 h, washed then stained for cell surface markers, CD69, HLA-DR, CXCR4, and CCR5.

3.5 Cell viability

Complimentary to the anti-HIV properties of 17BIPHE2, it was necessary to also ensure its lack of toxicity on target cells. Therefore, multiple cell viability assays were undertaken to verify the peptide's lack of toxicity to ACH-2, TZM-bl, PBMC, and CD4+ T cells.

3.5.1 Cell viability of ACH-2 cells post 2 h 17BIPHE2 incubation

Apoptosis and cell death were assessed by Annexin V and PI staining respectively. These stains were used to ensure ACH-2 viability (Figure 17). The number of Annexin V and PI positive cells did not change, remaining low in untreated cells and cells treated with 2.8, 5.6, or 11.2 µM 17BIPHE2 for 2 h indicating that 17BIPHE2 did not increase apoptosis or death of ACH-2 cells.

3.5.2 Cell viability of TZM-bl, PBMC, and CD4+ T cells post 2 h 17BIPHE2 incubation as measured by calcein-AM and PI

Calcein-AM and PI were used to assess TZM-bl viability, PBMC, and CD4+ T cell viability after cells were incubated with 17BIPHE2 for 2 h (Figure 18). Expression of fluorescent green calcein indicates viable cells with functional esterase activity. The number of calcein positive cells did not change, remaining at 75-85% of total cells in 0, 5.6, 11.2, or 22.4 µM 17BIPHE2 treated cells for all cell types. PI staining also did not change, thus indicating viability of the cells after 17BIPHE2 treatment of 2 h.

3.5.3 Cell viability of activated PBMC and CD4+ T cells post 2 h 17BIPHE2 incubation and subsequent 4-day incubation, evaluated with JC-1 dye

JC-1 is a dye used to indicate whether there is a change of the mitochondrial membrane potential during the peptide treatment. A decrease in the mitochondrial membrane potential, which results in a decrease of the red/green fluorescence ratio, signifies the loss of cell viability. CCCP, which disrupts mitochondrial membrane potential, was used as a positive control with which a decrease in the red/green fluorescence ratio was observed as compared to untreated cells (Figure 19). Cells were treated with 17BIPHE2 for 2 h, followed by a wash, then resuspension in medium containing IL-2. Cells were then further cultured for 4 days, mirroring the methodological timeline for HIV infection experiments. The representative dot plots of green versus red showed similar distribution between 0, 5.6, 11.2, and 22.4 µM 17BIPHE2 treated cells for all cell types (Figure 19 A&B).

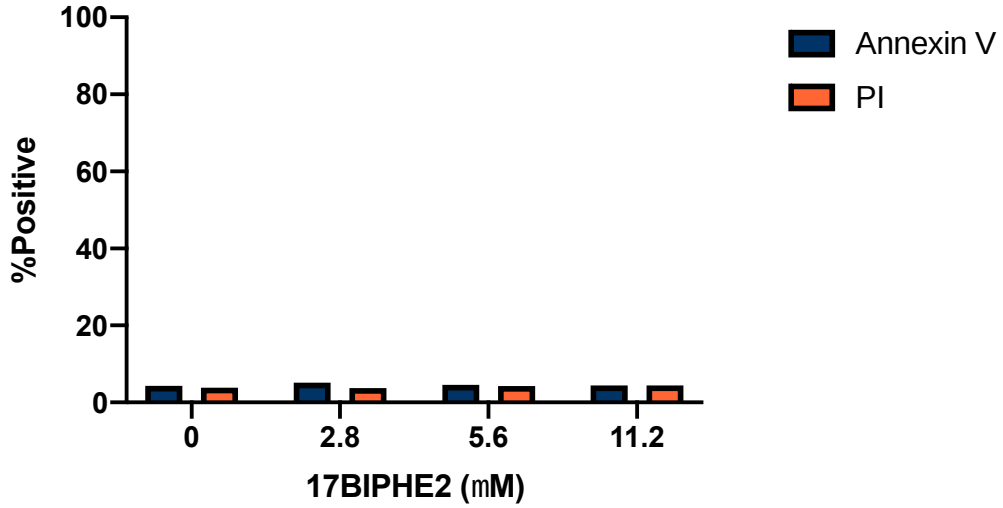
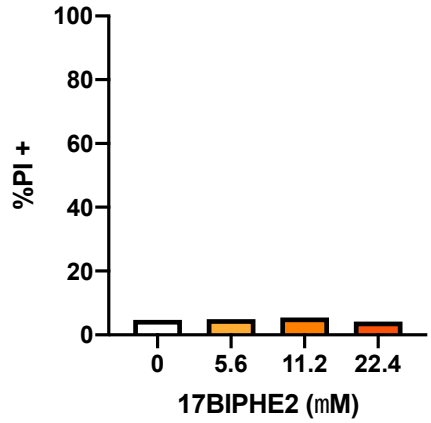
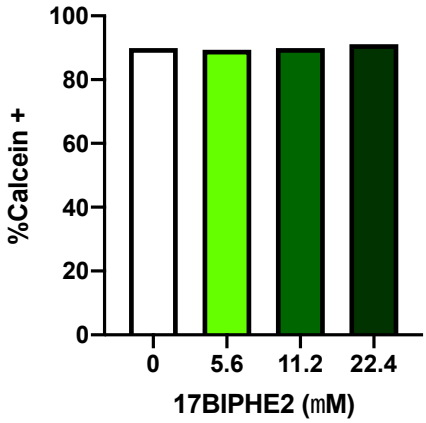

100
80
60
40
20
0
%Positive
0
2.8
5.6
11.2
17BIPHE2 (mM)
Annexin V
PI

Figure 17. ACH-2 viability.

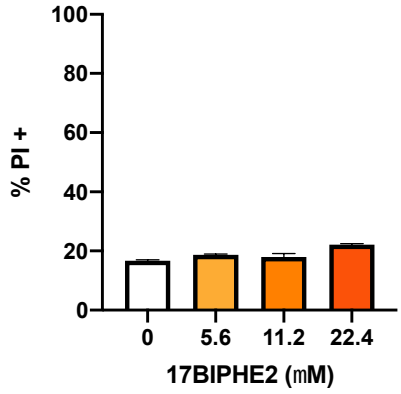
ACH-2 cells were incubated with 17BIPHE2 for 2 h followed by Annexin V and PI staining and analysis via flow cytometry. n=2

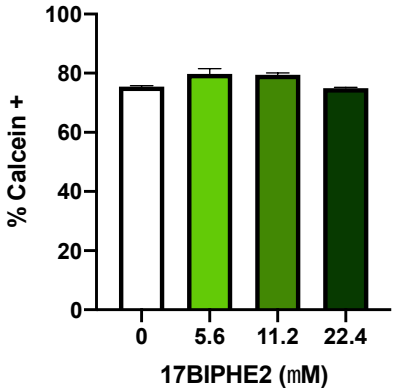
A


100
80
60
40
20
0
%PI +
0
5.6
11.2
22.4
17BIPHE2 (mM)

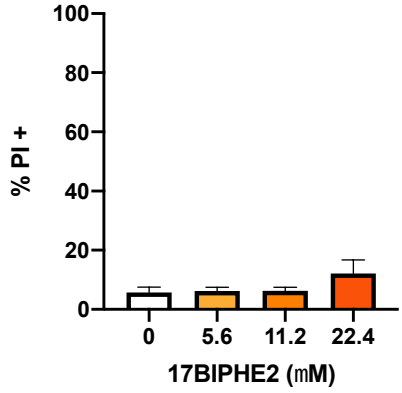

100
80
60
40
20
0
%Calcein +
0
5.6
11.2
22.4
17BIPHE2 (mM)

B


100
80
60
40
20
0
% PI +
0
5.6
11.2
22.4
17BIPHE2 (mM)


100
80
60
40
20
0
% Calcein +
0
5.6
11.2
22.4
17BIPHE2 (mM)

C


100
80
60
40
20
0
% PI +
0
5.6
11.2
22.4
17BIPHE2 (mM)

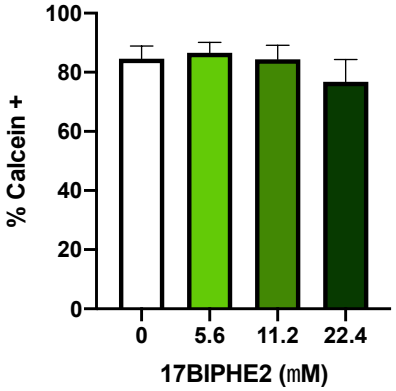
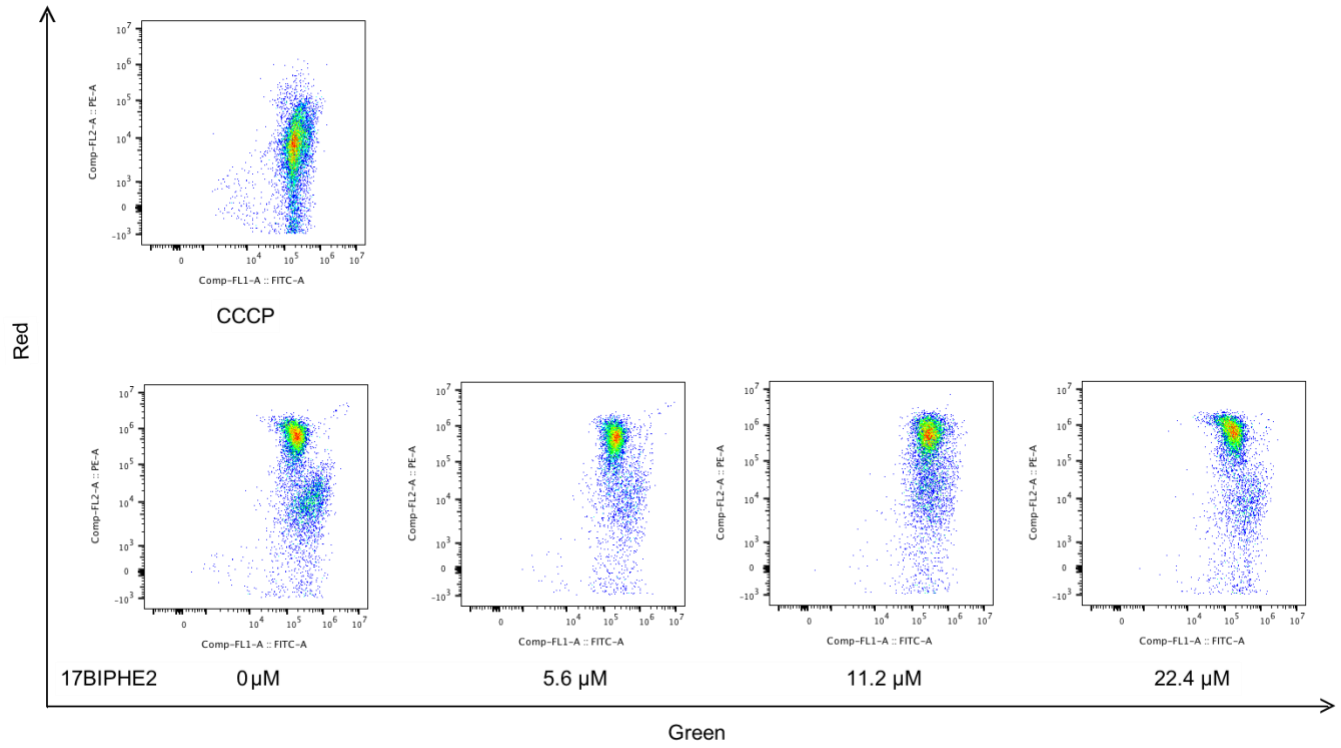

100
80
60
40
20
0
% Calcein +
0
5.6
11.2
22.4
17BIPHE2 (mM)

Figure 18. Viability via Calcein-AM and PI.

A) TZM-bl (n=2), B) activated PBMC (n=3), and C) activated CD4+ T cells (n=3) were incubated with 17BIPHE2 for 2 h followed by PI staining and Calcein-AM staining and analysis via flow cytometry. One-way ANOVA, ns.

A


Red
Green
Comp-FL2-A :: PE-A
Comp-FL1-A :: FITC-A
CCCP
17BIPHE2
0 µM
5.6 µM
11.2 µM
22.4 µM

B

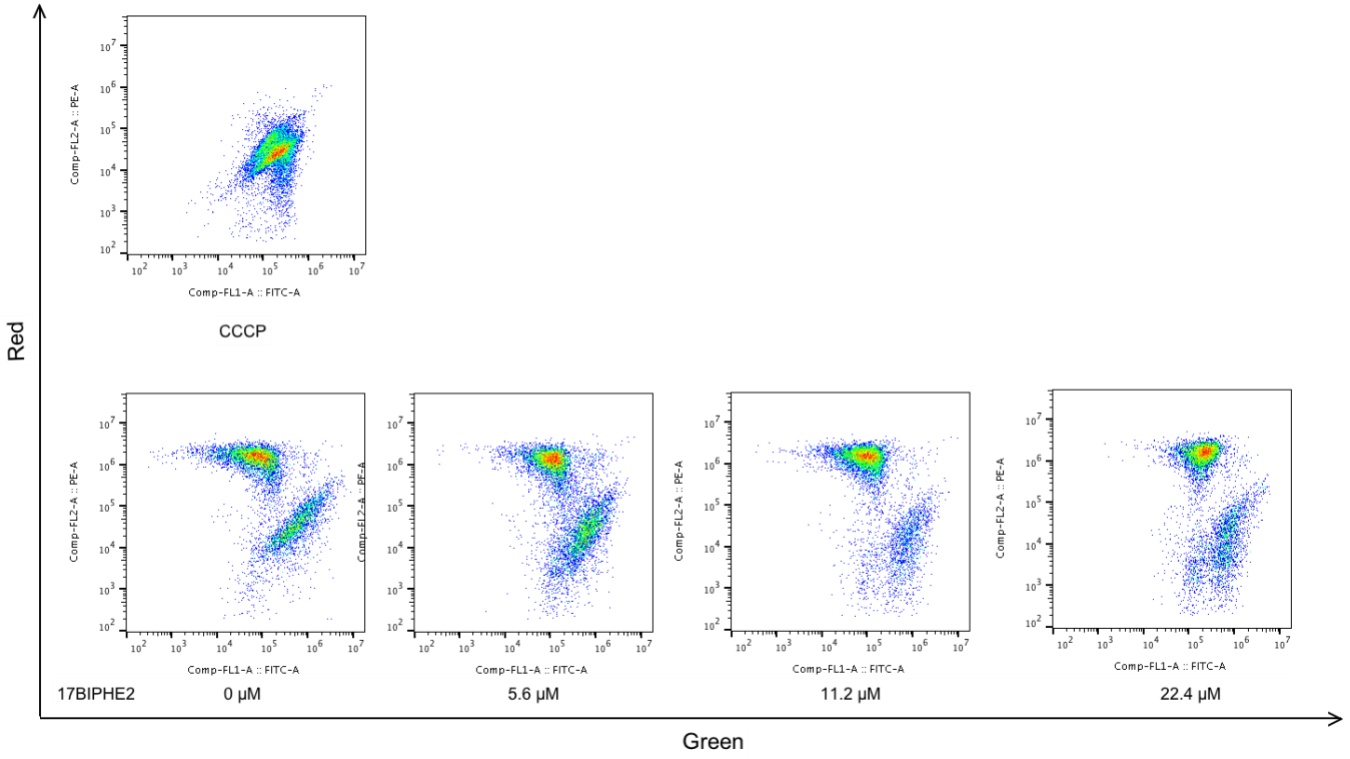

Red
Green
Comp-FL2-A :: PE-A
Comp-FL1-A :: FITC-A
CCCP
17BIPHE2
0 µM
5.6 µM
11.2 µM
22.4 µM

Figure 19. JC-1 Assay.

A) Activated PBMC (n=3) or B) CD4+ T cells (n=3) were incubated with 17BIPHE2 for 2 h, then washed and cultured for an additional 4 days. Cells were added with JC-1 and analyzed via flow cytometry. CCCP, a disruptor of mitochondrial membrane potential, was used as a positive control for metabolically compromised cells. Representative flow cytometry dot plots of three replicates show the distribution of JC-1 (red)-aggregates and JC-1 (green)-monomer populations. In general, 17BIPHE2 had no obvious impact on the JC-1 (red)-aggregates versus JC-1 (green)-monomer populations.

Chapter 4: Discussion

To summarize the findings of this project, the truncated engineered LL-37, 17BIPHE2, was able to inhibit HIV infection in both cell lines and primary cells. In the chronically infected cell line, ACH-2, 17BIPHE2 inhibited HIV replication whereas LL-37 did not. This inhibition of HIV replication gave reason to further pursue 17BIPHE2. 17BIPHE2 was also able to inhibit HIV infection of the TZM-bl reporter cell line when pre-incubated with HIV prior to infection and present during infection, but this result was not observed in the primary activated cells, PBMCs nor isolated CD4+ T cells. However, pre-treatment of target cells with 17BIPHE2 prior to infection significantly reduced HIV replication in a dose-dependent manner. Cell viability experiments indicated that treatment of cells with 17BIPHE2 did not cause toxicity, eliminating cell death as an explanation for such findings. Mechanisms of this inhibition must be further studied as preliminary studies involving measuring cell-surface markers of activation and co-receptor expression indicated no change between untreated and 17BIPHE2-treated cells.

4.1 17BIPHE2, but not LL-37, reduces HIV replication in ACH-2

As previously mentioned, LL-37 has been reported to reduce HIV infection in both cell lines and primary cells such as monocyte-derived dendritic cells, PBMCs and CD4+ T cells [47–49]. However, when the chronically infected T-lymphoblastic cell line ACH-2 were treated with either LL-37 or 17BIPHE2, 17BIPHE2 reduced HIV replication in a dose-dependent manner whereas LL-37 did not. The finding that LL-37 inhibits HIV infection has not previously been investigated in ACH-2, a cell line containing integrated proviral HIV. Generally, cell lines often differ from primary cells both morphologically and genetically [87,88]. ACH-2 were initially synthesized to study chronic infection and re-activation of viral replication [82,89]. The parental T-lymphoblastic cell line, A3.01, were infected with HIV and cloned such that the clone, ACH-2,

survived infection and produced and secreted low levels of reverse transcriptase and p24 into the culture supernatant [82]. A3.01 were derived from a lymphoblastic leukemia [89]. Though A3.01 express CD4, ACH-2 are CD4-negative as the majority of A3.01 cells that survived HIV infection did not express CD4 [82]. ACH-2 therefore differ from primary peripheral blood cells as they are an immortalized cell line manipulated to readily study chronic HIV infection. It is therefore conceivable that the mechanism of inhibiting viral replication in such cells may differ from that of the primary cells infected with HIV.

It has been reported that truncated peptides such as GF-17 are more efficient in inhibiting HIV infection than the LL-37 parental peptide [48]. Therefore, 17BIPHE2, a truncated and engineered peptide may also be more efficient in HIV inhibition. GF-17 was originally synthesized as an isolated active fragment of LL-37 that would be less costly to synthesize because of its shorter length [17,81]. However; similar to its parental peptide, GF-17 was susceptible to degradation by proteases such as chymotrypsin [81]. 17BIPHE2 was thus engineered to contain unnatural amino acids to make it more protease resistant while maintaining antimicrobial function. In fact, 17BIPHE2 has better microbicidal activity against certain bacteria such as *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia coli* compared to LL-37 and GF-17 [40,90]. 17BIPHE2 is also less susceptible to protease degradation specifically by proteases contained within cervicovaginal fluid, an important characteristic for MPT development [79,81]. Considering these data, 17BIPHE2 ultimately seems to be a better anti-HIV therapeutic and multipurpose prevention technology candidate than LL-37.

4.2 17BIPHE2 may not exert its anti-HIV activity on the virus directly

In a previous study, LL-37 was incubated with Influenza A virus to observe the effect of the peptide on the viral membrane [46]. Using electron microscopy, they showed that LL-37 caused viral membrane breaks and blebbing [46]. It is thus possible that LL-37 or 17BIPHE2 has similar effects on HIV. Though pre-treatment of the virus with the peptide showed a dose-dependent response in TZM-bl cells, this was not observed in primary cells. Therefore, the results obtained in this project did not indicate that the peptide acts directly on the virus to make it less able to infect target cells. Furthermore, simultaneous addition of virus and peptide on TZM-bl cells also decreased HIV infection indicating that 17BIPHE2 may not act directly on the virus, but interferes with the cell/virus interaction or acts on the cell itself. This is consistent with the results from Bergman and colleagues which found that pre-treatment of HIV with LL-37 did not result in an inhibition of HIV infection [47]. Though pre-treatment of HIV with 17BIPHE2 prior to infection of TZM-bl did result in a decrease in luciferase expression, the production of infectious virions in TZM-bl cells were not directly measured as they were in the primary cell experiments. It is also important to note that the titre used to infect primary cells was optimized to obtain sufficient p24 release into the supernatant for detection by ELISA whereas the titre needed in the TZM-bl experiments was much lower. The experiment was also performed in such a way that the peptide was not fully removed from the virus before infection of target cells. To better characterize whether the virus acts directly on the virus, additional experiments would be necessary. For example, electron microscopy could be used to visualize the viral envelope after treatment with 17BIPHE2. Experiments involving pre-treatment of HIV with 17BIPHE2 followed by removal of the peptide prior to infecting cells could also clarify the impact of the peptide on the virus. Additionally, the ability of 17BIPHE2 to inhibit HIV proteins could also be

explored as LL-37 was found to be capable of inhibiting recombinant HIV reverse transcriptase and protease which may lead to a reduction in HIV replication [50].

4.3 17BIPHE2 acts on the cell or the cell/virus interaction to inhibit HIV infection

Since the results obtained did not support that 17BIPHE2 acted on the virus directly, it was further investigated whether 17BIPHE2 acts on the cell or the cell/virus interaction to inhibit HIV infection. Pre-treatment of activated PBMC or CD4+ T cells with 17BIPHE2 resulted in a decrease in HIV replication. This demonstrated that 17BIPHE2 exerts its anti-HIV activity either by changing target cells to make them less susceptible to infection or by interfering with the cell/virus interaction to inhibit infection. This inhibition of HIV infection was not viral strain specific as two different strains, HIV_{CS204} (dual-tropic) and $HIV_{NL4.3}$ (X4-tropic) were used. This is also consistent with the findings from Bergman et al. (2007) in which pre-incubation of PBMC or CD4+ T cells with LL-37 resulted in an inhibition of HIV replication [47].

17BIPHE2 seemed to have a greater effect in PBMCs than isolated CD4+ T cells (Figure 14&15). Several factors may account for this result. Though lymphocytes account for the majority of PBMCs, other cells such as monocytes and plasmacytoid dendritic cells are also present [91]. 17BIPHE2 may act differently on these cells to reduce HIV replication to a greater degree than in isolated CD4+ T cells. Furthermore, LL-37 has been found to induce regulatory T-cell (Tregs) generation within PHA-activated PBMCs [65]. Tregs can suppress inflammation and viral dissemination[92], therefore generation of these cells may contribute to the anti-HIV activity of LL-37 and 17BIPHE2. Different viral strains were used in the PBMC and CD4+ T cell experiments, HIV_{CS204} and $HIV_{NL4.3}$ respectively. 17BIPHE2 may interact with these viral strains differently, resulting in varying degrees of viral inhibition.

4.4 Possible mechanisms of action

4.4.1 Co-receptor expression and markers of activation

The mechanism by which LL-37 and 17BIPHE2 inhibit HIV remains unclear. To preliminarily investigate possible mechanisms, expression of HIV co-receptors, CCR5 and CXCR4, were assessed after treatment with 17BIPHE2. Since either co-receptor is required for HIV entry into the target cell, differential expression of CCR5 or CXCR4 may change the propensity of the target cell to become infected. In the study by Ogawa et al. (2013), they reported upregulation of CCR5 in monocyte-derived Langerhans cells, but downregulation of CCR5 in monocyte-derived dendritic cells [49] in response to LL-37. The upregulation of CCR5 expression in mLCs was thought to contribute to their increased susceptibility to HIV infection after LL-37 treatment whereas the downregulation of CCR5 expression in mDCs may have decreased their susceptibility to infection [49]. Upon 17BIPHE2 treatment, neither CCR5 nor CXCR4 expression changed in CD4+ T cells. Though the functional activity of these receptors after 17BIPHE2 treatment has yet to be characterized, the peptide did not modulate their expression thus the inhibition of HIV infection in CD4+ T cells was not due to a decrease in co-receptor expression. It is possible that 17BIPHE2 binds these co-receptors and this binding was not captured by the experiment presented due to the short length of the monoclonal antibodies used. 17BIPHE2 may have bound to other areas on the co-receptors. LL-37 and 17BIPHE2 may also impede on the function of these co-receptors or interfere with the interaction between the co-receptors and the virus. As previously mentioned, the V3 loop of the HIV-1 gp120 glycoprotein is cationic [48,62]. Therefore, competition for binding to CCR5 or CXCR4 might occur between LL-37 or 17BIPHE2 and the V3 loop thus inhibiting HIV infection (Figure 1).

Markers of activation were also assessed as their differential expression may have also played a role in the observed decrease in HIV replication. A characteristic of untreated HIV infection is a heightened immune activation [93]. *In vivo*, markers of T-cell activation can be used as prognostic markers of disease progression [93,94]. Therefore, a potential mechanism of HIV inhibition would be decreasing immune cell activation to inhibit target cells from supporting productive infection. However, PHA and IL-2-activated CD4+ T cells treated with 17BIPHE2 showed no change in markers of activation, CD69 and HLA-DR, compared to untreated cells. The finding that 17BIPHE2 did not alter CD69 expression was consistent with the literature pertaining to LL-37, which upon treatment of PBMCs (resting and activated) did not change CD69 expression [65]. This indicated that the mechanism by which 17BIPHE2 inhibited HIV replication did not involve altering cell activation.

4.4.2 Immunomodulatory properties of LL-37/17BIPHE2 that may inhibit HIV replication

LL-37 is known to cause immunomodulation, potentially affecting HIV infection [45,49,64,66,92]. As previously mentioned, LL-37 can alter CCR5 expression (Ogawa et al. 2013), upregulate type I interferon signaling (He et al. 2018), and induce the generation of Tregs [65]. Alexandre-Ramos et al. (2018) demonstrated that LL-37 significantly increased transcription of pro-inflammatory cytokines such as TNF-α and INF-γ in resting PBMC cultures. In PHA-activated PBMCs stimulated with LL-37, TGF-β expression was significantly decreased. TNF-α and INF-γ expression also decreased though these results were not statistically significant. Such pro-inflammatory cytokines are highly involved in HIV pathogenesis and can promote viral replication and dissemination, therefore suppressing the release of these cytokines might explain an inhibition of HIV infection [95–97]. A number of cell surface receptors for LL-37 including formyl peptide receptor-like 1 (FPRL1), tyrosine kinase receptors such as the epidermal growth

factor receptor (EGFR), toll-like receptors (TLR) and the purinergic channel receptor P_2X_7R have been identified [43]. The immunomodulatory properties outlined above may, therefore, be mediated through receptor binding. The immunomodulatory properties of 17BIPHE2 however have not been characterized and although 17BIPHE2 exhibits similar microbicidal properties as LL-37[40], it is unclear whether 17BIPHE2 will also be capable of the same immunomodulatory activity.

4.5 Limitations and future directions

Though CD4+ T lymphocytes represent the main targets of HIV in the genital tract, peripheral blood-derived CD4+ T cells were used. These cells differ from CD4+ T cells in the female genital tract. For example, CD4+ T cells in the vagina highly express the co-receptor, CCR5 whereas peripheral blood CD4+ T cells express very little CCR5 (~14% of blood CD4+ T cells) and highly express CXCR4 [98,99]. Considering this limited CCR5 expression, peripheral blood CD4+ T cells were infected using an X4-tropic virus, $HIV_{NL4.3}$. In humans, transmitted HIV is usually CCR5-mediated [3], a factor that should be considered when interpreting the significance of these results. A more accurate model would involve obtaining CD4+ T cells from the female genital tract of donors.

Aside from further exploration into the mechanisms in which these AMPs inhibit HIV, other studies must be undertaken to further develop these peptides into anti-HIV therapeutics. An example of such studies would be to examine the effects of LL-37/17BIPHE2 in other HIV target cells. Cells within the female genital tract that represent initial HIV target cells include dendritic cells and Langerhans cells. As previously described, LL-37 has been shown to inhibit HIV infection in monocyte-derived dendritic cells, but increase HIV susceptibility in monocyte-

derived Langerhans cells [49]. This has yet to be examined in the case of 17BIPHE2 and should be done with dendritic cells and Langerhans cells collected from the female genital tract. Other studies have used an *ex vivo* organ culture system to further delve into the initial events of HIV infection in the vagina [3]. A similar system, such as MatTek EpiVaginal Tissue Model (human-like 3D tissue structure and cellular physiology), which are commercially available, could be deployed to further investigate how these AMPs might impact HIV-1 infection in a more physiologically relevant model.

The models used in this study did not include the vaginal microenvironment, including exogenous or endogenous hormones and the vaginal microbiome, all of which can impact HIV infection [100]. Though some of these variables could be included in *in vitro* and *ex vivo* models, *in vivo* animal models such as humanized mice and non-human primates would be another way to ensure a more holistic depiction of HIV infection.

Initial results concerning the safety of these peptides in mice have been promising. Mice were vaginally administered LL-37 or 17BIPHE2 and exhibited no histological damage to the reproductive tract nor induction of inflammatory cytokine production [72,73]. Mice also resumed fecundity once peptide treatment was halted [79].

The future of this work involves developing a delivery system to administer AMPs to the vagina for their use as preventative agents against HIV and other microbes as well as spermicides. However, formulating drugs for vaginal administration poses some challenges. The anatomy of the vagina encompasses a large opening to the external environment and contains mucin in the vaginal lumen which can trap administered compounds and impede them from reaching target cells [101,102]. Vaginal rings, gels, creams, ointments, suppositories and film strips

have thus been used as delivery systems to retain compounds within the vagina [103]. LL-37 has been formulated into creams and gels for clinical use [77,104,105] but has yet to be developed for vaginal administration Another intriguing delivery method for AMPs is nanotechnology, which has been developed for vaginal delivery of a number of microbicides [102,106,107].

4.6 Summary and significance

In summation, the findings of this project indicated that 17BIPHE2 can inhibit HIV when physiologically relevant target cells such as PBMCs and CD4+ T cells are pre-treated with this peptide prior to infection. 17BIPHE2 also did not induce toxicity to such target cells. Therefore, the results presented indicate that 17BIPHE2 may act on the cell or on the cell/virus interaction to inhibit HIV infection. The mechanisms of this inhibition remain unclear and must be further studied. Preliminary results involving measuring cell-surface markers of activation and co-receptor expression indicated no change between untreated and 17BIPHE2-treated cells suggesting that altering target cell activation nor changing co-receptor expression are not the mechanisms by which 17BIPHE2 acts.

This project has significant implications for MPT development as 17BIPHE2 also possesses spermicidal activity and is microbicidal against other sexually transmitted pathogens [79]. Women continue to represent a population at high risk of HIV in many regions of the world while other STIs, such as gonorrhea, chlamydia, syphilis, and herpes continue to compound the risk of HIV acquisition [108,109]. Concurrently, women are burdened with an unmet need for contraception, specifically women in lower- and middle-income countries [110]. The development of AMPs such as 17BIPHE2 into MPT therefore has the potential to significantly improve

women's health, potentially allowing them more options to control their sexual and reproductive health.

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