Development of an Infectious Cell Center Assay (ICCA) to Predict Appropriate Dosing of HIV Topical Microbicides

By

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DEDICATION

For My Family

Thank you for always believing in me when I don’t believe in myself.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AMFAR	American Foundation for AIDS Research
API	Active Pharmaceutical Ingredient
ARV	Antiretroviral
AZT	Azidothymidine / Zidovudine
BV	Bacterial Vaginosis
CD4	Cluster of Differentiation 4
CDC	Center for Disease Control
CPE	Cytopathic Effect
DNA	Deoxyribonucleic Acid
DPV	Dapivirine
dsDNA	Double Stranded Deoxyribonucleic Acid
EC	Efficacious Concentration
ELISA	Enzyme - Linked Immunosorbent Assay
FDA	Food and Drug Administration
FTC	Emtricitabine
H_2O_2	Hydrogen Peroxide
HAART	Highly Active Anti-Retroviral Therapy
HIV	Human Immunodeficiency Virus
ICCA	Infectious Cell Center Assay
IN	Integrase
IND	Investigational New Drug

INI	Integrase Inhibitor
INSTI	Integrase Strand Transfer Inhibitor
IVR	Intravaginal Ring
mL	Milliliter
mM	Millimolar
MOI	Multiplicity of Infection
MSM	Men who have sex with men
MTSA	Microbicide Transmission and Sterilization Assay
N-9	Nonoxynol-9
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NtRTI	Nucleotide Reverse Transcriptase Inhibitor
NNRTI	Nonnucleoside Reverse Transcriptase Inhibitor
OTC	Over the Counter
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
pH	Potential Hydrogen
PR	Protease Enzyme
PI	Protease Inhibitor
PrEP	Pre-Exposure Prophylaxis
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
RNA	Ribonucleic Acid
RT	Reverse Transcriptase

RTI	Reverse Transcriptase Inhibitor
SFU	Syncytium Forming Unit
ssRNA	Single Strand Ribonucleic Acid
STI	Sexually Transmitted Infection
SQR	Saquinavir
TAF	Tenofovir Alafenamide Fumarate
TDF	Tenofovir Disoproxil Fumarate
TFV	Tenofovir
UNAIDS	United Nations Programme on HIV/AIDS
US	United States

ABSTRACT

With millions of people currently infected with human immunodeficiency virus (HIV), and with increasing rates of transmission around the world, the development of new and more potent HIV inhibitors is necessary. Along with development of new agents to treat existing infections, it is also important to consider products and methods to reduce the rates of transmission of HIV and prevent new infections from occurring. In light of the increasing burden of HIV infection in women especially in developing areas of the world, as well as the high transmissibility of HIV through anal sex with both women and men who have sex with men (MSM), one of the important types of products being developed are topically applied microbicides. Microbicides are formulated for use at the site of infection, so they must deliver a high enough concentration of inhibitor to be effective but be delivered without the possibility of unnecessary and potentially dangerous toxicity and systemic exposure. A critical requirement of microbicides is that the preventative agent must be present at the correct place (the site of infection) at the correct time and at the correct dose to prevent transmission of HIV.

This research project was designed to determine if an *in vitro* assay designated the Infectious Cell Center Assay, or ICCA, could be used to determine the optimal and correct tissue concentration of a microbicide product which would be required to effectively prevent transmission from occurring in the vaginal or rectal epithelial tissue. It is hypothesized that the prevention product must totally inhibit the initial transmission and infection event such that a subsequent spreading infection from the locus of initial infection does not occur. Thus, we propose to utilize the ICCA to define the sterilizing concentration of a microbicide product and utilize this information to prioritize products for continued development in the microbicide pipeline.

In developing the ICCA, the research performed effectively confirmed that the ICCA is able to determine the concentration of an antiretroviral (ARV) that is needed at the tissue level to prevent transmission and infection of HIV and thus result in sterilization of the cell culture. The ICCA was found to be more predictive than other *in vitro* assays due to the ability of the technology to quantify the number of infected cells present in each culture through the syncytium forming unit (SFU) assay. The ICCA was able to be used with various wild-type and drug resistant strains of HIV-1. To mimic the results of microbicide use by people, the effect of virus multiplicity of infection, drug pretreatment and delayed drug addition of compound were also simulated in the ICCA and defined as critical assay variables with importance to the development and use of microbicide products. The antiviral effect of combinations of antiretroviral agents was also able to be quantified in the ICCA. Most importantly, the ICCA defines sterilizing concentration of a microbicide product was able to be harmonized with concentrations of these same products shown to be effective in both *ex vivo* and *in vivo* research, suggesting that the *in vitro* defined sterilizing concentration is an important addition to the development of any topical microbicide product. We believe that with additional investigation, the ICCA might be validated as a more predictive *in vitro* assay used to define appropriate effective dosing concentrations of ARVs to be used as microbicide products in order to totally suppress HIV transmission and to prioritize products for further development in highly expensive and time consuming human clinical trials.

INTRODUCTION

HIV Epidemiology

The number of people infected with human immunodeficiency virus (HIV), the causative agent of acquired immune deficiency syndrome (AIDS), continues to increase worldwide despite significant efforts to combat the virus (amFAR 2019). According to the American Federation for AIDS Research (amfAR 2019), there are more than 37 million people currently living with HIV/AIDS worldwide. In 2018, there was an estimated 1.7 million new infections, with 5,700 new infections occurring every day, and 770,000 individuals annually succumbing to an AIDS related illness. One of the high-risk populations of the world is in sub-Saharan Africa, where more than 70% of the HIV-infected population resides, equating to approximately 21 million infected individuals and where 800,000 new infections occurred in 2018 alone (CDC 2018). Over half of the infected individuals in Sub-Saharan Africa are heterosexual women (amfAR 2019). Other high-risk areas include Asia and the Pacific, the Caribbean, Central and South America, North Africa and the Middle East (CDC 2018).

In the United States (US), the CDC estimates that there are more than 1.1 million people currently living with HIV, and that approximately 1 in 7 of these individuals are unaware of their infection status (amfAR 2019). The most seriously affected population by HIV in the US are men who have sex with men (MSM) (amfAR 2019). MSM in the US comprise approximately 4% of the male population, but accounted for 83% of new HIV infections, as well as 59% of all infected individuals in the US in 2018 (amfAR 2019). Young African Americans are disproportionally affected by HIV when compared to other races, but Caucasian MSM have accounted for the largest number of new

infections in recent years (CDC 2018). African Americans make up roughly 12% of the US population, but account for 44% of new HIV infections each year and make up 41% of people currently living with HIV in the US. Hispanics and Latinos are also increasingly affected by HIV in comparison to other races (CDC 2018). Hispanics and Latinos make up approximately 16% of the US population and account for 21% of new infections annually and 30% of the population currently living with HIV (CDC 2018). The CDC reports that the number of people living with HIV in the US has increased, whereas the number of new HIV infections has remained stable at around 50,000 new infections per year (CDC 2018). In total, there have been over 658,000 deaths of people with an AIDS diagnosis in the US since AIDS was defined in the 1980s (CDC 2018).

The most affected population by HIV after African American men is African American women. In total, African American women account for 19% of the population of newly infected people in the United States, and 23% of the total infected US population (CDC 2018). Of these new infections in the female population, 87% were attributed to heterosexual sex, and the remainder to injection drug use (CDC 2018). In this population, 62% are African American women, 18% are white women and 16% are Hispanic/Latino women (CDC 2018).

HIV Transmission

HIV is predominately found in genital secretions such as semen, seminal plasma and vaginal fluid, as well as in breast milk. HIV has also been found in small amounts in various other bodily secretions such as saliva, urine and tears although these secretions appear to be clinically irrelevant regarding transmission of the virus (Fauci and Desrosiers 1997). Less common methods of transmission that contribute to the total HIV-

infected population include sharing of tainted needles, mother-to-child transmission and blood transfusions (CDC 2018). The most common route of HIV transmission is through unprotected vaginal and anal sexual intercourse (CDC 2018). Infection by HIV through vaginal or anal sexual intercourse occurs after a mucosal inoculation event, where CD4+ and CCR5-expressing cells present in the epithelium of the vagina, rectum or foreskin are susceptible to HIV infection. Infection occurs in cells expressing CD4 and/or CCR5 cell surface receptors such as T- and B-cells, monocytes, macrophages, dendritic cells, endothelial cells and Langerhans cells. In heterosexual couples practicing unprotected, vaginal sexual intercourse, the risk of infection is higher for the woman than it is for the man, due to the virus being harbored in semen (CDC 2018). Some reports have suggested that the components of semen as well as the stage of infection of the infected partner may enhance HIV infectivity and transmission (Fox and Fidler 2010). That being said, virus is also harbored in vaginal and rectal fluids, but the more sensitive tissue to HIV transmission during sexual intercourse is the vagina and rectum in comparison to the penis. This is primarily due to structural differences in the protective epithelial layers of these tissues. The single columnar epithelial layer of the rectum results in significantly greater risk of virus transmission compared to the multilayered epithelial tissue of the vagina (Yu and Vajdy 2010). Other biological mechanisms, such as the greater degree of epithelial damage which occurs in in the vagina and rectum during sexual intercourse as well as the larger anatomical surface in the vagina with increased presence of target cells also explains the higher rates of transmission that are seen in male to female transmission versus female to male transmission (Fox and Fidler 2010). Lastly, higher rates of symptomatic sexually transmitted infections (STIs) that occur in females in comparison

to males leads to increased risk of infection in women compared to men (Fox and Fidler 2010). For example, women with active, ulcerative STIs such as herpes simplex virus (HSV) are at increased risk for HIV infection because the ulcers disrupt the vagina's mucosal barrier and increases the presence of inflammatory HIV target cells (Fleming and Wasserheit 1999). Non-ulcerative STIs, such as bacterial vaginosis, gonorrhea or syphilis, have also been correlated with increased risk of HIV infection in women due to the disruption of the natural bacterial flora present in the vagina and the influx of inflammatory cells which are primary targets for HIV infection (Martin *et al.* 2009).

In addition to biological factors increasing transmission of HIV through sexual contact, there are also behavioral factors that affect transmission. One of these is the number and nature of sex acts. A higher number of sexual partners and higher frequency of sexual contact have been associated with increased transmission of HIV (Fox and Fidler 2010).

Once the virion enters the CD4+ T-cell and goes through the process of uncoating to release the virion core into the cytoplasm, the viral enzyme reverse transcriptase initiates reverse transcription which transcribes the single stranded RNA viral genome into double stranded viral DNA. The newly synthesized closed circle double stranded DNA complex is transported through the nuclear membrane and integrates into the host genome through processes mediated by the HIV integrase protein. Following integration, the HIV provirus becomes an integral part of the host cell genome for the life of the infected cell and coordinates the viral transcriptional and translational processes, which results in the production of viral regulatory and structural proteins and viral enzymes which are then packaged into progeny viral particles. Virus buds from the plasma

membrane of the infected cell, undergoes viral protease enzyme mediated polypeptide cleavage and the mature infectious virion is then able to infect neighboring CD4+ cells (Gomez and Hope 2005). The HIV replication cycle is shown below in Figure 1. The loss of CD4+ T-cells degrades the effectiveness of the immune system slowly over time and when an individual has a CD4+ T-cell count of less than 200 cells/mm^3, patients are clinically diagnosed with AIDS (NIH 2016). After the onset of AIDS, the body is unable to fight off opportunistic infections and cancers, which ultimately, results in death.

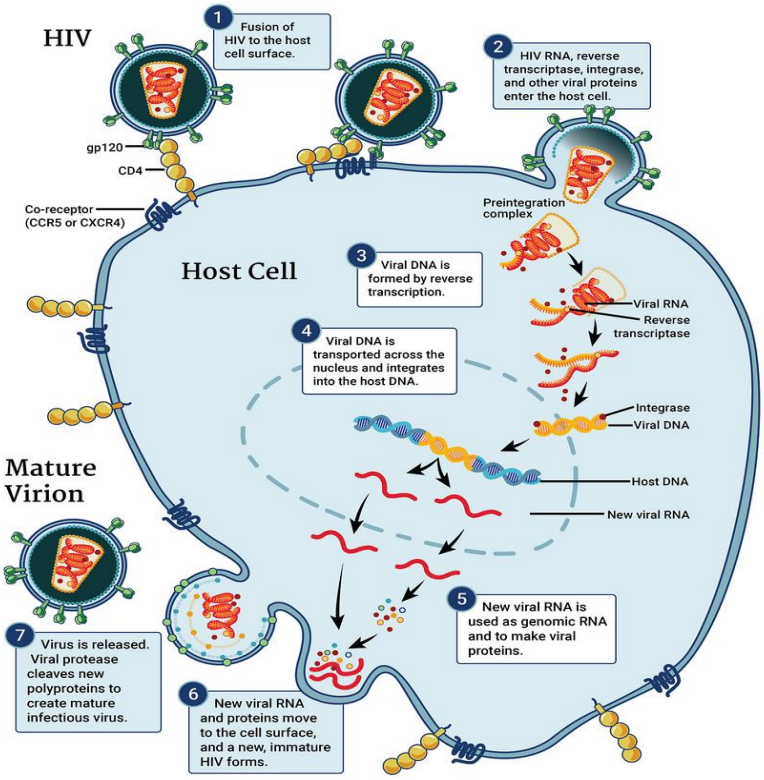


Figure 1. The replication cycle of HIV in a host cell (NIAID 2018)

HIV Pathogenesis

The development of AIDS from initial infection with HIV has several stages. Viral load is used to determine the rate that the immune system is being destroyed, while the number of CD4+ cells shows the degree of immunodeficiency and can be used to determine the stage of infection (Simon *et al.* 2006). Viral load and CD4+ count are also measured to monitor the impact of therapeutic strategies and the success of antiviral treatment (Simon *et al.* 2006).

The first stage of HIV primary infection occurs 3 to 6 weeks post infection and involves flu like symptoms (Fauci and Desrosiers 1997). During this time viral proteins and infectious virus can be detected in the plasma and cerebrospinal fluids and CD4+ T-cells also decline. Three to six weeks after the primary infection, there is a decline in plasma viremia and CD4+ T-cell numbers begin to stabilize due to an active host immune response (Fauci and Desrosiers 1997). CD8+ cytolytic T-cells appear during the immune response to maintain the number of lymphocytes and cause a decrease in plasma viremia. During this time clinical flu-like symptoms caused by the HIV infection disappear (Fauci and Desrosiers 1997).

The second stage of HIV infection, known as the clinical latent stage of infection, is asymptomatic with few clinical manifestations (Fauci and Desrosiers 1997). During this latent period, the CD4+ T-cell numbers continue to decline in the presence of continuous viral replication, which causes a steady increase in viral load. Eventually, the T-cell count falls below 500 cells/mL, and the clinical manifestations of AIDS begin to occur (Fauci and Desrosiers 1997). These clinical manifestations include tiredness, swollen lymph nodes in the neck and groin, fever, unexplained weight loss and

unexplained bruising or bleeding (Fauci and Desrosiers 1997). This second stage of infection can last up to 12 years in the absence of antiviral treatment, depending on the individual, but with current antiretroviral (ARV) therapies the gap in life expectancy between HIV infected and HIV uninfected individuals has been reduced to only 8 years (Fauci and Desrosiers 1997; Marcus *et al.* 2016).

The third and last stage of HIV infection is clinical AIDS. AIDS is diagnosed once the CD4+ count falls below 200 cells/mL, and common opportunistic infections that define AIDS begin to occur (Fauci and Desrosiers 1997). These opportunistic infections include Kaposi's Sarcoma (a skin cancer), fungal infections such as *Candida albicans* which colonize mucous membranes and *Pneumocystis jirovecii* which causes pneumocystis pneumonia, aseptic meningitis, and dementia (CDC 2018*)*. T-cell levels continue to decline until there are no detectable levels and the individual succumbs to one of the opportunistic infections listed above.

Treatment and Prevention of HIV

Treatment for those individuals infected with HIV consists of ARVs which decrease viral load and slow or prevent the onset of AIDS (Arts and Huzuda 2012). The first monotherapy ARV to obtain FDA approval was Zidovudine (azidothymidine or AZT), due to its effectiveness in reducing viral load and delaying disease progression (Fischl *et al.* 1987). AZT is a nucleoside reverse transcriptase inhibitor (NRTI) which inhibits HIV replication by terminating viral DNA strand synthesis through competitive binding with the reverse transcriptase enzyme (RT). Since the development of AZT in the late 1980s, over two dozen inhibitors targeting various steps in the virus life cycle have been approved by the FDA as treatments for HIV (FDA 2019). Combination

therapy is the current accepted strategy for the treatment for HIV infection, using approved inhibitors with different mechanisms of action to reduce viral load to undetectable levels and significantly decrease disease progression while suppressing the emergence of resistant viruses. These ARVs inhibit the entry and/or replication of the virus, resulting in undetectable levels of HIV in most infected individuals. However, HIV therapy is not curative, and the virus is not completely cleared from the body due to the provirus remaining an integral part of all infected and long-lived cells of the immune system (Arts and Huzuda 2012). The presence of HIV in cells in long term sanctuary sites, or viral reservoirs, within the body consequently results in the necessity for the patient to be on life-long treatment. Treatment of infected individuals leads to longer and healthier lives in comparison to those individuals infected prior to the availability of ARVs. Side effects and the toxicity of existing drug treatment regimens and the necessity for strict adherence to drug regimens to prevent a rebound of virus replication or the appearance of drug resistant virus makes long term therapy challenging (Arts and Huzuda 2012). It is also well known that ARVs are toxic to normal cells and tissues in the body, causing adverse side effects to those taking them. Short term side effects can include fatigue, nausea or diarrhea. Long term side effects can include cardiovascular disease, kidney failure, liver damage diabetes and loss of bone density (CDC 2018). Despite this, the use of highly active anti-retroviral therapy (HAART) and the development of better ARVs have resulted in HIV becoming more characteristic of a chronic disease, similar to diabetes (Arts and Huzuda 2010).

The development of a vaccine to prevent the transmission of HIV would undoubtedly be the best option for decreasing the demographic of individuals who are at

risk of infection. However, there currently is no FDA approved vaccine and though many clinical studies for vaccine development continue, a vaccine is still decades away from approval. The lack of a vaccine, despite intense efforts to create one, can be mostly attributed to the genetic diversity that is seen in the viral genome resulting from errors in transcription by the RT, as well as the ability of HIV to mask critical epitopes from the immune system through glycosylation of the HIV envelope glycoprotein.

In the absence of a cure for HIV or a vaccine to prevent HIV infection, other methods of inhibiting virus transmission are key to decreasing the number of newly infected individuals. Although social awareness of HIV transmission and knowledge of how to prevent its spread has had some impact on the rates of transmission, the number of new infections continues to increase in some demographic areas (amfAR 2018). For this reason, other methods of prevention are being developed. A primary focus of educational resources is used within communities with the highest risk of HIV infections. These high-risk communities include MSM and people at risk in Africa due to the pandemic spread of HIV. In the homosexual community, education on safer sex practices is provided, such as condom use, because the practice of receptive anal intercourse is a primary mode of HIV transmission. A study performed by Pinkerton *et al.* in 1997 showed that condom use alone is 90 to 95% effective in preventing HIV transmission and people who use condoms consistently are 10 to 20 times less likely become infected with HIV compared to inconsistent and non-condom users (Pinkerton and Abramson 1997). In Africa, more resources are being used to better educate individuals on the dangers of HIV and how it is contracted. African school systems are also encouraged to promote sex education and education on the dangers of needle sharing

and intravenous drug use. All high-risk individuals are also encouraged to get testing to determine their HIV status. In 2017, it was estimated that as many as 9.4 million people infected with HIV did not know their status (UNAIDS 2018). Earlier diagnosis results in earlier initiation of treatment, which has a direct impact on reducing the transmission of HIV (UNAIDS 2019).

In contrast to social strategies, pharmaceutical prevention of HIV transmission and infection involves the use of ARVs or other chemical agents to provide a chemical barrier to suppress or eliminate the transmission of HIV and to decrease or eliminate the likelihood of an individual contracting HIV. Chemical barrier prevention methods include agents such as pre-exposure prophylaxis (PrEP) and the use of topically applied microbicides. In the absence of an effective vaccine to prevent HIV infection, PrEP and vaginally or rectally applied topical microbicides rise to the forefront as a means to prevent virus transmission. PrEP is a powerful HIV prevention tool for people who are at high risk of being infected with HIV that can be combined with condoms to provide greater protection than one method alone (CDC 2018). These high-risk individuals take a daily regimen of orally dosed ARVs which accumulate in target tissues within the body and act as a barrier to prevent HIV transmission from an infected individual leading to subsequent virus replication and a spreading HIV infection. Currently, two PrEP products have been approved by the FDA. The first is Truvada, which is a combination of the approved ARVs tenofovir disproxil furmarate (TDF) and emtricitabine (FTC). Truvada was approved by the FDA in 2012 for use as a PrEP agent. In human clinical trials a 75% reduction in infection was observed and adherence to the drug strategy was high (Baeten *et al.* 2012). The second approved PrEP product is Descovy, which is a

combination of tenofovir alafenamide fumarate (TAF) and emtricitabine. Descovy was approved in October of 2019. The results from the DISCOVER Phase 3 clinical trial suggested Descovy acts similarly to Truvada in reducing the risk of HIV infection (Hunt 2019). This trial enrolled 5,400 MSM or transgender women who have sex with men (Hare, Smith 2019). Of the individuals participating in the study, 7 HIV infections were identified in individuals using Descovy as oral PrEP, and 15 infections were identified in individuals using Truvada (Hare, Smith 2019). Descovy unlike Truvada, is not approved for use in women since its effectiveness against vaginal HIV transmission has yet to be assessed (Hunt 2019).

Topical Microbicides

In addition to PreP, vaginally and anally delivered microbicides are also a method of prevention of HIV. Topical microbicides are defined as chemical agents (drugs) that are applied inside the vagina or rectum and are capable of preventing the transmission of HIV or result in direct inactivation of microorganisms, such as HIV or other STIs at the site of infection. Microbicides have been developed in many formats including semi-solid gels, creams, films, tablets, intravaginal rings, and suppositories. These products can act in different ways to prevent the transmission of these pathogens. Some products provide a physical/chemical barrier through entry inhibition mechanisms so that the pathogen never reaches the target cells to initiate infection. Development of these entry inhibiting microbicides in general has been disappointing (reviewed in Shattock and Rosenberg 2012). For example, the entry inhibitor PRO2000, a synthetic polymer which was shown to have antiviral activity against HIV-1 and other STIs *in vitro* and *in vivo* (McCormack *et al.* 2010) was tested for its ability to prevent HIV transmission in human

clinical trials. A Phase III randomized clinical trial (MDP 301) was performed in Africa to determine the efficacy and safety of a 0.5% and 2% PRO2000 gel in women (McCormack *et al.* 2010). Although the gel was determined to be safe for use in humans and antiviral activity was seen in macaques, the 0.5% and 2% PRO2000 gel were not effective in preventing vaginal transmission of HIV, even with adherence being high. For this reason, the study was discontinued prior to the end of the study (McCormack *et al.* 2010).

In addition to physical barriers, other microbicides have been developed that enhance the natural defense mechanisms within the vagina by maintaining an acidic vaginal pH, which has been shown to protect from infection in the vagina (Bouvet *et al.* 1997). Hydrogen peroxide (H_2O_2) produced by vaginal lactobacilli has been shown to prevent infection from HIV and other STIs (O'Hanlon *et al.* 2013). It is hypothesized that increased production of H_2O_2 by these bacteria reduces the incidence of infection caused by *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, HIV-1 and HIV-2 in the vagina (O'Hanlon *et al.* 2013). A study performed by Martin *et al.* showed a correlation between the absence of vaginal *Lactobacillus* colonization in the vagina and an increased risk of HIV-1 infection, bacterial vaginosis (BV) and gonorrhea in sex workers in Kenya (Martin *et al.* 2009). Women lacking *Lactobacillus* were shown to have a 2.5-fold increased risk of acquiring HIV compared to women with H_2O_2 producing lactobacilli, although presence of any lactobacilli, H_2O_2 producing or not, appeared to be beneficial (Martin *et al.* 2009).

Another example of enhancing the natural defense mechanisms of the vagina with *Lactobacillus* is the engineering of *Lactobacillus jensenii*, a bacterium naturally found in

the vaginal flora, to express neutralizing antibodies against the glycoproteins present on the surface of the HIV envelope (Marcobal *et al.* 2016). *In vitro*, these bacterially expressed antibodies were shown to bind to the gp120/CD4 complex, specifically the CD4-induced binding site (CD4), which results from the conformational change in gp120 as it binds to the CD4 (Marcobal *et al.* 2016). By binding to the CD4, the antibody inhibits the second binding of the CCR5 co-receptor and thus prevents virus attachment (Marcobal *et al.* 2016). This use of a natural enhancement microbicide would benefit HIV prevention by delivering antibodies where virus first enters the body (Marcobal *et al.* 2016). Lactobacilli have also been engineered to produce and secrete a natural antiviral product which acts as an entry inhibitor (cyanovirin-N) (Boyd *et al.* 1997).

Microbicides containing ARVs have been shown to be the most successful in terms of preventing HIV infection. Tenofovir, the first and most successful topical microbicide to date, inhibits the replication cycle of the virus by preventing reverse transcription (Abdool *et al.* 2010). In a Phase 3 human clinical trial (CAPRISA 004), a 1% tenofovir microbicide gel was shown to reduce HIV-1 infection by 39% compared to a placebo gel used by high risk women in Africa (Abdool *et al.* 2010). This study provided proof of concept that a microbicide containing an ARV can impact the transmission of HIV in an at-risk population of women (Abdool *et al.* 2010). In the Phase III ASPIRE trial, a vaginal ring delivering the non-nucleoside reverse transcriptase inhibitor dapivirine resulted in a 37% reduction in HIV-1 infection in women compared to a placebo ring (Baeten *et al.* 2016). However, despite the continued development of multiple delivery mechanisms of microbicides, none of these modalities have proven to be 100% effective. Although lack of adherence by the user contributes to the lack of

efficacy, the ineffectiveness could also be attributed to not having the right drug, in the right place, at the right dose, at the right time.

Not all microbicide formulations and delivery methods for HIV prevention are practical for all women. For example, the use of condoms is not practical when a woman wants to conceive a child or has a partner that will not use one (Abdool 2010). Women have clear preferences for the types of products that they will want to put into their bodies and these products need to match the woman's requirements for such a product when having sex. Microbicides, however, enable women to protect themselves when some practical methods of HIV prevention are not applicable. Microbicides are designed to be used daily, around the time of coitus, or in slow release formulations that provide protection over a prolonged period of time. Since the microbicide formulations are created for topical use, higher concentrations of drug can be delivered without the risk of systemic exposure which might result in greater toxicity or insufficient drug concentrations which might result in the selection of drug resistance upon infection (reviewed in Shattock and Rosenberg 2012).

Prioritizing Microbicide Candidates

There are many important characteristics which must be taken into account when microbicide products are being prioritized for clinical trials and eventual use. Besides efficacy against HIV, the most important characteristic involved in prioritizing microbicide candidates is safety. Since microbicides are developed for use in uninfected, healthy individuals, the product should be locally nontoxic. This is critical for topical microbicide use because it is important to avoid utilizing a microbicide which causes damage to the vaginal or rectal epithelium resulting in portals of virus entry which

enhance or facilitate virus transmission. It is also important to assure that a microbicide product does not have toxicity to naturally occurring Lactobacilli strains, which have natural antiviral protective properties. Safety of a microbicide also includes evaluation of systemic toxicity, long term toxicity, and potential impact on fertility and fetal development as well as any irritation to the tissues of the vagina, rectum or penis (Chasela *et al*. 2010).

The second important characteristic for microbicide prioritization is the efficacy associated with a microbicide candidate, meaning that the microbicide must have a significant, preventative effect on the transmission of HIV to uninfected individuals. It has been predicted that a single microbicide with 60% efficacy would have the potential to prevent over 1 million new HIV infections per year (Watts *et al.* 2002). However, there is controversy over the level of protection that is necessary for a microbicide to be considered efficacious, though the potential proof of concept for effective microbicide use is supported by data generated from the CAPRISA 004 and ASPIRE trials which each reported 40% reduction in HIV transmission.

Another important consideration for the development of effective microbicide products involves the fact that many of the ARVs being developed as microbicide products have already been used to treat HIV (reviewed in Shattock and Rosenberg 2012). The use of these same products as topical microbicides could become a significant problem if the chemical barrier to infection permitted the transmission of a resistant virus to the microbicide. Furthermore, infection with the resistant virus would impact potential therapeutic options for the now-infected individual in the future (reviewed in Shattock and Rosenberg 2012). Another consideration for product

development is the potential for resistant virus selection if use of a microbicide product is continued after infection occurs since topical microbicide use would most likely result in low suboptimal systemic exposure to the ARV, allowing for potentially rapid resistant virus emergence. The likelihood/risk of these events occurring is unknown but it must be hypothesized that the use of microbicide products which have been used therapeutically and to which resistant virus populations have occurred and are present in the circulating HIV species population would allow for resistant strains of virus to easily breach the microbicide barrier (reviewed in Shattock and Rosenberg 2012). The potential for resistance to emerge from a topically used microbicide that is not associated with substantial systemic exposure has not been explored (reviewed in Shattock and Rosenberg 2012). However, it is important to note that in the CAPRISA 004 study, there was no evidence of a tenofovir-resistant virus emerging from subjects who became infected during the trial (Abdool *et al.* 2010). Since the transmission of resistant isolates is possible, the use of a microbicide product based on a single ARV may be inadvisable because the resistant isolate will be not inhibited by a drug to which it is resistant (reviewed in Shattock and Rosenberg 2012). A second (or multiple) drug in the microbicide product may provide a more effective barrier to transmission in the same way that combination therapy is more effective than single agent monotherapy.

Appropriate and optimized drug delivery is another important characteristic when prioritizing microbicides. Appropriate drug delivery requires sufficient drug levels to be maintained at the target areas, such as the vagina or the rectum. Obviously, this is more difficult to accomplish with coitally dependent products when compared to slow release formulations (Romano *et al.* 2008). In the CAPRISA 004 study where a coitally

dependent microbicide gel was used, the higher reported adherence to the dosing regimen correlated with higher reduction in HIV transmission (Abdool *et al.* 2010). In this study, high adherence to the dosing regimen (> 80%) showed a 54% reduction in HIV transmission, moderate adherence (50 to 80%) showed 38% reduction and low adherence (< 50%) showed 28% reduction compared to the placebo (Abdool *et al.* 2010). Overall, the 1% tenofovir gel prevented HIV transmission by an estimated 39%, with a median adherence level at 61.3% (Abdool *et al*. 2010). Ease of drug delivery also has been shown to impact the adherence to a microbicide dosing regimen, which ultimately effects the efficacy of the product. In the ASPIRE Phase 3 clinical trial, which assessed the efficacy of a dapivirine ring, adherence was shown to be high, with greater than 80% of plasma samples collected containing concentrations of dapivirine associated with intravaginal ring (IVR) use (Baeten *et al.* 2016). As in the CAPRISA 004 trial, higher degrees of adherence correlated with higher reduction in HIV transmission (Baeten *et al.* 2016). Based on the results of these clinical trials, it can be determined that drug delivery via topically applied gel or intravaginal ring can be beneficial options for microbicide delivery and adherence may be dependent on individual preferences.

Another key characteristic in the prioritizing of microbicides is the cost to manufacture large quantities of the product. This is an issue mainly because individuals in the most at risk populations are unable to afford these products. Thus, microbicide products must be developed and manufactured with cost in mind to maximize their potential utility and assure widespread use (Romano *et al.* 2008). A cost-benefit analysis has shown that even introducing a microbicide with low effectiveness to the most at risk

countries could substantially reduce HIV infection, which would reduce health care costs by billions of dollars (Romano *et al.* 2008).

Although it is apparent that microbicide use will be beneficial, many promising microbicides are unable to be tested due to the time, labor and cost of the necessary preclinical development and human clinical safety and efficacy trials needed to bring these products to market for approved use. Due to the nature of the ethical human clinical trials which must be performed, human clinical trials will require hundreds of millions of dollars per study and will need to recruit over 10,000 at risk uninfected participants. Thus, human clinical studies for advancing products will need to be highly prioritized for candidate products with the highest possibility of clinical success. To determine the best in class products and to expedite development of highly active ARVs suited for microbicide development, appropriate and highly sensitive and discriminating *in vitro* evaluations are required to determine potency and toxicity of promising candidates.

Microbicide Development

Early Development of Microbicides

Microbicides were originally intended to be products offering protection from a broad range of STIs, using compounds that would be available without a prescription (reviewed in Shattock and Rosenberg 2012). Research on HIV microbicides started with products already containing some antiviral activity, such as spermicides. Spermicides, such as over the counter (OTC) surfactant nonoxynol 9 (N-9) were shown to impact the structural integrity of HIV *in vitro*. Following the Phase III clinical trial of N-9 as a microbicide, it was determined that surfactant-based products were not only unable to

prevent HIV infection, but when used frequently disrupted the vaginal epithelium to a degree where higher rates of infection were seen (Hillier *et al.* 2005).

After the failure of N-9 as a microbicide, other strategies for OTC microbicide products were evaluated. Such strategies included lowering the pH of a product to inactivate HIV. This strategy was based on a study in 1995, showing infectivity levels of HIV decreasing when pH levels fell below 4.5 (O'Connor *et al.* 1995). Clinical trial results showed that this method was safe but was not efficacious enough to prevent infection.

After failed attempts at finding an OTC product capable of inhibiting HIV infection, efforts were moved to repurposing potent HIV specific approved and effective ARVs into prevention agents targeting one of four areas of replication: entry, reverse transcription, integration or virus maturation (Ramjee and McCormack 2010). The effectiveness of a microbicide relies on the pharmacokinetic ability of ARVs to get to the appropriate site of infection at the right time and at the right dose and thus products must be chosen and formulated carefully.

Targeting Viral Entry

Viral entry into target cells is the first step in the virus life cycle where microbicides are able to effect virus transmission. There is a relatively short window of opportunity for microbicides to block infection since the attachment and entry of a virion into a target cells occurs in minutes to a couple of hours after exposure (Murray *et al.* 2011) In studies performed in nonhuman primates, HIV was shown to establish an infection after a 30 to 60 minute exposure to an infectious virus inoculum (reviewed in Shattock and Rosenberg 2012). CD4 binding on target cell surfaces is necessary for viral

entry, but there are few compounds effective in blocking the gp120-CD4 interaction (Vermeire *et al.* 2008). Further, CD4 on T-cells is known to have important functions in driving T -cell response, and the impact of suppression of CD4 expression in regard to immunity and immune reactivity, is unknown. A less potentially harmful target would be the gp120 on the surface of the virion to prevent necessary interactions with the target cell surface CD4 (Si *et al.* 2004). Studies show a small protein that mimics the T-cell CD4 and will block infection *in vitro* (Si *et al.* 2004). Alternatively, studies show that small-molecule compounds, such as BMS-806 are able to bind gp120 and prevent envelope conformational changes required for attachment and virus entry, rendering the virion unable to infect the T-cell. Other inhibitors include peptides created to mimic the gp41 sequence which prevents fusion of the viral and cellular membranes, such as the approved drug Fuzeon. Although these approaches are possible, the cost of manufacturing and creating stable formulations of a protein-based microbicide able to remain efficacious in mucosal environments is a challenge (Si *et al.* 2004).

Another means to inhibit viral entry is to target co-receptor binding to prevent fusion of the viral envelope to the host cell. Of the two co-receptors used in viral fusion, CXCR4 and CCR5, epidemiological evidence shows CCR5 expressing viruses result in greater than 90% of new acute HIV infections across the globe (Gaertner *et al.* 2008). Thus, targeting CCR5 is a good strategy to prevent initial infection. CCR5 antagonists bind the transmembrane domains of CCR5, altering the trans-membrane domains which prevents gp120 binding (Gaertner *et al.* 2008). Drugs targeting CCR5 include regulated on activation normal T cell expressed and secreted (RANTES) protein analogs and other small molecule inhibitors (Gaertner *et al.* 2008). Currently, Maraviroc is the only CCR5

inhibitor that is safe and approved for use in humans as an antiviral therapy (Gaertner *et al.* 2008). Maraviroc is able to be manufactured with established processes and has been widely studied in clinical trials, which makes it a candidate for microbicide development for entry inhibitor-based microbicides.

Targeting Reverse Transcriptase

There are two classes of reverse transcriptase inhibitors (RTIs) that have been approved for use in the treatment of HIV infection which might have utility has topical prevention agents. The first class of reverse transcriptase inhibitors are nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs). Nucleoside inhibitors were the first class of drugs to be developed to combat HIV (Broder, 2010). NRTIs work by competing with the natural nucleosides to be incorporated into the newly synthesized viral DNA. After the virus enters the host cell, the reverse transcriptase converts the single strand RNA into double stranded DNA using nucleotides. When NRTIs are incorporated into the elongating viral DNA (in place of natural nucleosides) they cause termination of the elongating DNA strain, interrupting DNA synthesis and therefore viral replication. Nucleotide RT inhibitors work by the same mechanism, but their structure is different from the nucleoside analogs in that they are already phosphorylated. Tenofovir, the first NtRTI tested as a microbicide, resulted in promising data in a Phase 3 human clinical trial (Abdool *et al.* 2010).

The characteristics of tenofovir make it a suitable choice for development as a microbicide. Tenofovir requires two phosphorylation steps before becoming active, while other nucleoside inhibitors such as AZT require three (Rohan *et al.* 2010). One of the most notable characteristics about the active form of tenofovir is its tissue half-life, which

can be up to 50 hours intracellularly and is one of the longest half-lives seen in the NRTI class (Anderson *et al.* 2010). The tissue half-life of a drug is the time required to reduce by 50% the peak concentration of an active drug in the tissue and is widely used pharmacokinetic parameter used in drug development. The longer the half-life in the tissue, the larger the window of protection, which is ideal for a microbicide. The tenofovir prodrug, TDF, is also ideal for development as a microbicide, also due to the prodrug entering cells more rapidly as well as its long intracellular half-life of 150 hours (Anderson *et al.* 2010). Currently, tenofovir is the only NRTI being developed as a microbicide and it has been approved for use as a component of the Truvada PrEP regimen mentioned above (Faria *et al.* 2019). To date, there are currently seven licensed NRTIs that are being used alone and in combination in HAART and other forms of PrEP.

The second class of RTIs are nonnucleoside reverse transcriptase inhibitors (NNRTIs). These drugs inhibit HIV viral DNA synthesis by noncompetitive inhibition of the reverse transcriptase. In order for the RT to synthesize viral DNA from the RNA template, the RT enzyme itself must change conformation. NNRTIs attach to a binding pocket on the RT, rendering it incapable of binding to the RNA preventing DNA synthesis and viral replication. NNRTIs are known to show high affinity for binding the RT enzyme, making them useful therapeutic agents (reviewed in Shattock and Rosenberg 2012). Two products which are utilized in the project reported herein, Dapivirine and IQP-0528, are examples of NNRTIs being developed as microbicides. NNRTIs may be better suited for microbicide development than NRTIs due to NNRTIs not requiring phosphorylation steps to become active and they are freely diffusible, meaning they enter cells quickly and freely to inhibit virus replication. NNRTIs remain in their inhibitory

active states (no metabolism or modifications required) so they do not have the lag times associated with NRTIs to achieve efficacious concentrations in the cell.

Targeting Viral Integration

Integration occurs through the integrase (IN) enzyme which works to insert a copy of the transcribed circular DNA into the host DNA forming genomic proviral copy of the HIV genome. Once the proviral DNA is integrated into the host genome, it becomes an integral component of the host cells and is replicated along with other cellular genes, providing a template for the viral RNAs needed to synthesize more virus (Cragie and Bushman 2012). Preventing integration is the key to preventing an irreversible infection. Ideally, microbicide products should exert their antiviral effect prior to integration of viral DNA into the host cell genome to prevent infection (Turpin, 2002). Using integrase inhibitors (INIs) alone as topical microbicides have significant drawbacks, being that the mechanism of action of INIs occurs late in the replication cycle of HIV (Crucitti *et al.* 2012). The virus must first enter the host cell and reverse transcription must occur before the INI can act (Crucitti *et al.* 2012). Because of this, it is recommended that INIs be used in combination with ARVs that act earlier in the replication cycle of HIV (Crucitti *et al*. 2012). Due to these limitations, there is currently little information or data on the utility of INIs as a microbicide, in combination or alone although raltegravir has been evaluated as a microbicide product. Currently, there are 4 INIs with potent HIV activity that are being used therapeutically, including, raltegravir, dolutegravir, elvitegravir and bictegravir.

Targeting Viral Maturation

The protease in the HIV replication cycle is responsible for cleavage of the Gag and GagPol precursor proteins. These precursor proteins, when cleaved, give rise to the structural proteins and the viral enzymes reverse transcriptase, integrase and protease. When the precursor proteins are cleaved correctly, formation of mature and infectious virus particles is possible (van Maarseveen and Boucher 2006). Most protease inhibitors (PIs) are designed to mimic natural substrates targeted by the viral protease and bind to the active site of the enzyme. By blocking the active site, the protease is unable to cleave the Gag and GagPol precursor proteins correctly, causing non-infectious viral particles to be released (van Maarseveen and Boucher 2006).

Currently, there are ten licensed PIs that are being used as HIV therapeutic products. Protease inhibitors are interesting candidates for microbicide use because of their high barrier for resistance (Stefanidou *et al.* 2012). Reports of resistance against PIs has been noted, mainly because of the error prone RT resulting in mutants with decreased sensitivity to protease inhibitors (van Maarseveen and Boucher 2006). PIs were not initially considered for microbicide development due to their effect late in the HIV replication cycle, but data suggest that ARVs that act later in the HIV replication cycle are able to show greater inhibition of virus production than early acting ARVs (Stefanidou *et al.* 2012). Because PIs act late in the virus replication cycle, it can be hypothesized that although PIs cannot inhibit integration, that by targeting viral proliferation and inhibiting the release of infectious virus, PIs could clear infection as the founder population of infected cells die (Stefanidou *et al.* 2012). Through this mechanism, PIs could be a useful addition to a combination microbicide product with an

ARV that acts at an earlier stage in the replication cycle of HIV, such as tenofovir or dapivirine (Stefanidou *et al.* 2012). To test this theory, a study performed by Stefanidou *et al.* in 2012 determined the potency of the PI saquinavir *in vitro* and *ex vivo*. Although saquinavir was shown to be efficacious in multiple *in vitro* cell lines, unfortunately these results were not reproduced when HIV inhibition was tested in genital explant cultures where saquinavir showed no significant inhibition of infection (Stefanidou *et al.* 2012). Though saquinavir was unable to prevent infection, it was able to inhibit the release of infectious virus when the infected explants were co-cultured with uninfected T cells (Stefanidou *et al.* 2012). In addition, darunavir was evaluated as a component of a combination product intravaginal ring along with dapivirine (Murphy *et al.* 2014). These data suggest that a single PI as a microbicide is not recommended, but with further investigation, the addition of PIs to combination microbicide products could be beneficial.

Combination Microbicides

Currently, most microbicides being developed are single agent ARV microbicides, but research on developing microbicides containing two or more ARVs is ongoing (reviewed in Shattock and Rosenberg 2012). Combination products utilizing dapivirine (a NNRTI) and maravoric (CCR5 blocker) in an intra-vaginal ring have shown success in Phase I clinical trials (Chen *et al.* 2015). However, it was determined that maraviroc concentrations were only detectable in the cervicovaginal fluid unlike dapivirine which was able to penetrate the plasma, suggesting the maraviroc is not released well from the ring (Chen *et al.* 2015). The International Partnership for Microbicides also states that the development of vaginal rings containing dapivirine in

combination with DS003 (gp120 inhibitor) and dapivirine combined with darunavir are being developed (IPM 2019). By adding ARVs with different mechanism of action to a single microbicide product, multiple stages of the HIV replication cycle can be targeted, increasing the efficacy of the product. A single ARV microbicide is able to be circumvented by resistant isolates, rendering it useless. By adding a second ARV to the microbicide, the microbicide would remain effective. Secondly, the combination of ARVs in microbicides might allow for lower concentrations of compounds to be used with increased potency against the virus and decreased potential for toxicity (reviewed in Shattock and Rosenberg 2012). Thirdly, adding compounds targeting different stages of the virus replication cycle provides a greater chance of protection (Sturt 2010).

Formulation Strategies

Initially, the concept for microbicide development was the application of a gel close to the time of sexual intercourse (coitally dependent dosing). There are advantages and disadvantages associated with this formulation strategy. The advantages of a gel formulation include the gel providing a large quantity of drug directly before and after possible virus exposure, as well as being a self-controlled method of protection. The disadvantages of a gel formulation include adherence by the individuals using the product. If the individual forgets to use the product, there is a higher risk of infection. In addition, with coitally dependent products people will have to anticipate when they may have sex, which is also not always practical (Turner *et al.* 2009). To deal with this uncertainty, some dosing strategies require daily use of the gel, independent of sex, in the hope that daily dosing would provide a steadier state of drug levels, as well as increase compliance (reviewed in Shattock and Rosenberg 2012).

In addition to microbicides being used as creams or gels, products have also been developed as intravaginal rings like those that are used with certain birth controls and hormone replacements (Malcom *et al.* 2010). Microbicide impregnated rings could be used to steadily release drugs over long periods of time. This was evaluated in the ASPIRE clinical trial, discussed above, using the NNRTI dapivirine in steady release conditions at one-month intervals, with rings being discarded and replaced every 4 weeks (Baeten *et al.* 2016). This study demonstrated that intravaginal rings were highly acceptable as a drug delivery method among individuals in high risk populations and are able to be worn safely for a month or longer with little to no adverse side effects (Baeten *et al.* 2016).

The premise of vaginal ring development is that the ring will be able to stably deliver optimal concentrations of drug that is required to be protective over a longer period of time and drug delivery is not coitally dependent. A disadvantage to this method is that the ring may not release as high of a dose of drug as observed with a daily application of a cream or gel. However, adherence with the use of an intravaginal ring product should be higher because there is no self-dosing regimen required (Romano 2008). However, in the ASPIRE trial, adherence was not as high as initially predicted, especially in young women (age 18 to 21) participating in the trial (Baeten *et al.* 2016). This low adherence is thought to be associated with the age of the participants, the potential product side effects, user perceptions and acceptance of the product format, and the participants concern about the safety and efficacy of the product (Baeten *et al.* 2016).

Microbicide Dosing

Understanding the dosing of a microbicide product and how this relates to the overall effectiveness of the microbicide has become a key focus in the development of new microbicide products. Historically in the microbicide field, the strategy for dosing HIV prevention products has been to dose tissues with the highest, non-toxic concentration that is possible, as determined by *in vivo* safety studies. This strategy is perpetuated as a direct result of the lack of data from successful human clinical trials, or data from a predictive animal model with direct correlation to humans. There are risks associated with this strategy as well, such as product side effects and toxicities.

Research in our laboratory has particularly focused on defining an in vitro solution to this dosing and tissue concentration scientific question. We initially developed a Microbicide Transmission Sterilization Assay (MTSA) to assist with identifying required tissue concentrations of microbicide products that would totally sterilize a culture of HIV (Watson *et al*. 2008). The MTSA was developed to more appropriately mimic the sexual transmission of HIV *in vitro*, relative to other assays that are included in microbicide development algorithms, as well as to better define the concentration of a candidate microbicide which would totally suppress the replication of virus in target cells and tissues (Watson *et al*. 2008). Thus, the data obtained from the MTSA defines the concentration of a microbicide in cells and tissues which would be necessary to completely prevent the transmission of virus to these target cells (Watson *et al*. 2008). There are, however, limitations to the MTSA including the labor-intensive nature of the assay as well as the length of time required to obtain results (45 days). In contrast to the MTSA, the newly developed Infectious Cell Center Assay (ICCA) was developed to

more accurately determine the concentration of a microbicide that would sterilize a target cell culture from virus transmission and infection. Compared to the MTSA, the ICCA is better able to mimic the events of sexual transmission of HIV and allows quantification of results of sterilization in a shorter amount of time, with less labor intensity and greater sensitivity. With the introduction of the ICCA, we hope to correlate the *in vitro* sterilizing concentration of a microbicide to the sterilizing concentration required at the site of infection in both *ex vivo* and *in vivo* models. From these data correlations, we hypothesize that the ICCA will be able to predict the dosing of a microbicide product that will be required in order to achieve sterilizing concentrations of the product in uninfected target cells and tissues.

As described above, the perpetuation for the current dosing strategies of microbicides is the result of a lack of data from a successful clinical trial for a microbicide candidate. It may be presumed that the lack of complete prevention in the clinical trials performed to date may in fact be evidence of incomplete sterilization of virus in the *in vivo* setting, although the lack of adherence to the dosing regimens for the products adds significant complexity to understanding the results of the clinical trials. At the time of initiation of the development of the ICCA we had no correlative data from animal models or humans that we can relate to our *in vitro* sterilization assay results. To begin to provide a link between *in vitro, ex vivo* and *in vivo* studies in both animals and humans, the ICCA will need to provide insight into accurate correlation of the *in vivo* dosing and concentrations of products required in target cells and tissues from *in vitro* and *ex vivo* studies. Understanding the amount of a drug that is required to completely suppress virus transmission and infection and being able to use this information to

accurately predict the dosing required will be a significant advancement to the microbicide development field.

MATERIALS AND METHODS

Cell lines and Viruses

The CEM-SS cells (Nara, Hatch *et al*. 1987), HIV-1_{IIIB} (Popovic *et al*. 1984), HIV-1_{RF} (Otto *et al*. 1995), HIV-1_{A17} (Nunberg *et al*. 1991) and the pNL4-3 used to generate infectious HIV-1_{NL4-3}, HIV-1_{Y181C} and HIV-1_{K103N} were obtained from the NIAID AIDS Research and Reference Reagent Program (Adachi *et al.* 1986). The CEM-SS cells were stored in the gas phase of liquid nitrogen and the viruses were stored at -80°C.

Antiviral Agents

IQP-0528 was synthesized at and made available by Samjin Pharmaceutical Company Ltd. (Seoul, South Korea). Dapivirine was gifted to ImQuest BioSciences from the International Partnership for Microbicides (IPM) and tenofovir was gifted to ImQuest BioSciences from Gilead Sciences. IQP-0528 and dapivirine were solubilized in DMSO at 1 mM and 40 mM and tenofovir was solubilized in water at 10 mM. All compounds and drugs were stored at 4°C. Structures of compounds are shown below in Figures 2A-C.

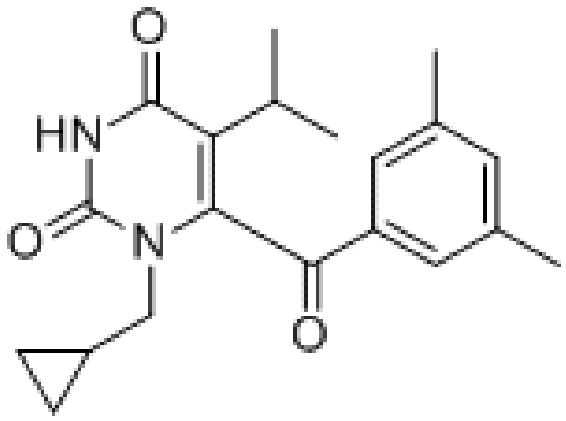


Figure 2A. 2D Structure of non-nucleoside reverse transcriptase inhibitor IQP-0528 (DrugBank)

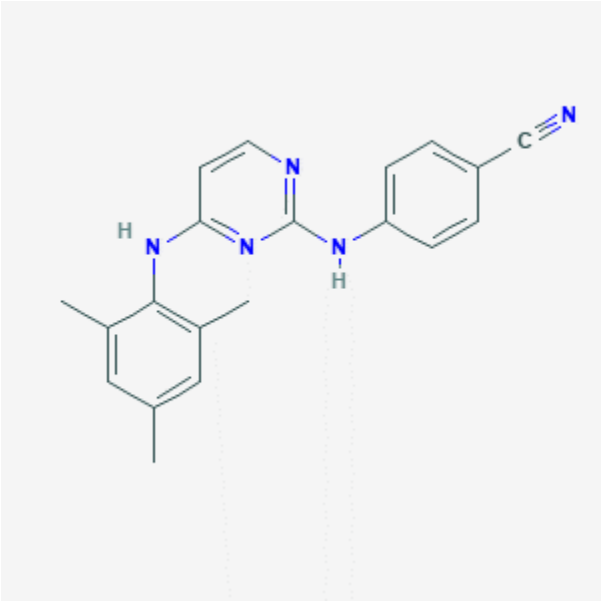


Figure 2B. 2D Structure of non-nucleoside reverse transcriptase inhibitor dapivirine 4-[[4-(2,4,6-trimethylanilino)pyrimidin-2-yl]amino]benzonitrile (Pubchem)

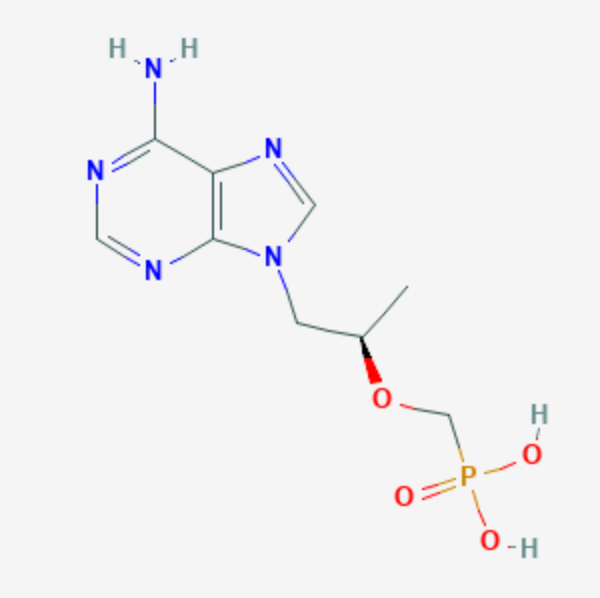


Figure 2C. 2D Structure of nucleoside analog reverse transcriptase inhibitor tenofovir (R)-9-(2-Phosphonomethoxypropyl)adenine (Pubchem)

Cell Culture

The CEM-SS suspension cell line used in these studies was propagated in RPMI-1640 media (Lonza, Walkersville, MD) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS)(Gibco-Life Technologies., Gaithersburg, MD), 2 mM L-glutamine, 100 Units/mL penicillin and 100 mg/mL streptomycin (Lonza). This media is referred to as complete RPMI-1640. This cell line was cultured and maintained in a 37°C, 5% CO_2 humidified incubator. The CEM-SS cells were maintained at a cell density between 1 x 10^5 and 1 x 10^6 cells/mL. The day before assay initiation, the CEM-SS cells were sub-cultured 1:2 to ensure that they were in the exponential growth phase by the time of the assay. New CEM-SS cells were propagated following 22 to 25 passages.

Cell Density and Viability Determination

The cell density and viability of the cultures was determined by staining the cells with trypan blue (Sigma Aldrich, St. Louis, MO). After re-suspending the CEM-SS cells, 15 µL of the cell suspension was removed from the total cell suspension and added to 15 µL of 0.4% trypan blue. After combining the cell suspension and trypan blue, 15 µL was added to a hemocytometer and covered with a glass cover slip. Cells were observed under 100X magnification on an inverted microscope. The total number of cells was counted in the four outside squares of the grid on the hemocytometer. Unstained cells were counted as live cells while stained cells were counted as dead cells. The total viable cell population was calculated by multiplication of the live cells by 1×10^4 and the dilution factor (0.5). The percent viability was calculated by dividing the number of live cells by the total number of dead cells and multiplying by 100%.

Preparation and Storage of Virus Pools

After the number of viable cells in the CEM-SS cell culture was determined, 5×10^5 cells were transferred to a 50 mL conical tube (Thermo Fischer, Waltham, MA) and centrifuged at 1000 rpm for 10 minutes. The supernatant was removed from the conical tube, and the cell pellet was re-suspended in 100 µL of complete RPMI-1640. The cell suspension was transferred to a 96 well, U-bottomed microtiter plate (Thermo Fisher). One hundred microliters (100 µL) of cell free virus at the appropriate titer was added to the well, for a final in-well volume of 200 µL. The plate was then placed in a humidified incubator at 37°C and 5% CO_2 for 1 hour. Following the incubation, the infected CEM-SS cells and viral supernatant were transferred to a 25 cm^2 flask (Corning, Corning NY) containing 5 mL of complete RPMI-1640. The cultures were then incubated at 37°C/ 5%

CO_2 for 24 hours at which time an additional 5 mL of complete RPMI-1640 was added, bringing the total volume in the flask to 10 mL. Each day following the initial infection, the cultures were monitored for syncytium formation (described below). The cell density and viability were calculated before the centrifugation of the cell suspension at 1000 rpm for 10 minutes. Following centrifugation, virus containing supernatant was stored at -20°C for reverse transcriptase (RT) activity assay and other analysis. The remaining cell pellet was re-suspended in 10 mL of complete RMPI-1640 and transferred to a 25 cm^2 flask and returned to the incubator. This process was repeated daily until the cell viability of the cells in the flask was below 50%. The frozen supernatant samples were then thawed and evaluated for RT activity using a biochemical RT assay (described below). The level of activity was directly proportional to the amount of virus in the sample. The daily supernatant pool with the highest level of RT activity was considered the peak day of the pool and was further distributed into 1 mL aliquots and stored at -80°C.

Cell Observations and Morphology During Infection

The cultures were observed under 40X view using an Olympus CKX31inverted microscope to evaluate the stage of HIV infection in each culture. The evaluation of infection was based on the amount of syncytium formation seen in the cultures. Clumping cells and newly formed syncytia were suggestive of the early stages of HIV infection with some syncytia showing a "ballooning" appearance. Peak of infection was identified by many large syncytia in most of the cell population. After the viral peak, balloon degeneration and the presence of dying, granular syncytia denote a well-advanced viral infection. The syncytia formation was rated on a semi-quantitative, visual

scale of +1 to +5, with the increasing numbers indicating more syncytia and observable cell death.

Reverse Transcriptase (RT) Activity Assay

Reverse transcriptase was measured in cell-free supernatants generated in the ICCA using a standard radioactive incorporation polymerization assay. Tritiated thymidine triphosphate (TTP) (Perkin Elmer, Waltham MA) was used at 1 Ci/mL, and a volume of 1 μL was used per enzyme reaction. Poly rA (Sigma-Aldrich, St. Louis, MO) and oligo dT (Affymetrix, Santa Clara, CA) were prepared at concentrations of 0.5 mg/mL and 1.7 Units/mL, respectively, from a stock solution which was stored at -20°C. The RT reaction buffer was prepared immediately prior to the assay and consisted of 125 μL of 1 mol/L EGTA (Sigma-Aldrich), 125 μL of dH_2O, 125 μL of 20% Triton X-100 (Sigma-Aldrich), 50 μL of 1 mol/L Tris (Sigma-Aldrich)(pH 7.4), 50 μL of 1 mol/L DTT (Gold Biotechnology, St. Louis, MO), and 40 μL of 1 mol/L $MgCl_2$ (Sigma-Aldrich). For each reaction, 1 μL of TTP, 4 μL of dH_2O, 2.5 μL of poly rA:oligo dT (rAdT) and 2.5 μL of reaction buffer were mixed. Ten microliters (10 μL) of this reaction mixture was transferred to a round bottom microtiter plate and 15 μL of virus-containing supernatant was added and mixed. The plate was incubated at 37°C for 60 to 90 minutes. Following the incubation, 10 μL of the reaction volume was spotted onto a DEAE filter mat (Perkin Elmer) in the appropriate plate format, washed 5 times in approximately 50 mL of a 5% sodium phosphate buffer (Fisher Scientific) for 5 minutes each, 2 times for 1 minute each in deionized water, 2 times for 1 minute each in 100% reagent alcohol and then the filter mats were allowed to air dry. The dried filter mat was placed in a plastic sleeve (Perkin

Elmer) and 4 mL of Opti-Fluor O (Perkin Elmer) scintillation fluid was added to each sleeve. The filter mat containing pouch was placed in a 96-well cassette holder and read on a Microbeta Trilux (Perkin Elmer), with each well being counted for 15 seconds. Incorporated tritiated thymidine monophosphate radioactivity in each 15 µL sample was quantified. Data obtained from the RT activity assay were presented as counts per 15 seconds.

Cytopathic Effect (CPE) Inhibition Assay

The cytopathic effect (CPE) inhibition assay was used to determine efficacious and toxic concentrations (EC and TC, respectively) of the antiviral compounds used in the ICCA (described below). Compounds were serially diluted in half-log increments and added to a 96-well round bottom plate (Falcon) in triplicate in a volume of 100 µL to the appropriate wells. CEM-SS cells were plated in the appropriate wells at a density of 2.5×10^3 cells/well in a volume of 50 µL. Virus was diluted to a pre-determined titer and added to the plate in the appropriate wells in a volume of 50 µL. Controls for each compound included compound colorimetric control wells (compound only), compound cytotoxicity control wells (cells and compound), virus control wells (cells and virus only), and cell viability control wells (cells only). The plates were then incubated at 37°C/5% CO_2 for six days. Following the incubation, 50 µL of XTT [2,3-bis(2-methoxy-4-nitro-5-sulfonpenyl)-2H-tetrazolium-5-carboxanilide] (Sigma Aldrich) tetrazolium salt and phenazine methosulfate (PMS) (Sigma Aldrich) solution was added to all wells of the plate. The XTT- tetrazolium dye is converted by mitochondrial enzymes in viable cells to form a colored product. The XTT solution was prepared immediately before being added to the plate by solubilizing 1 mg/mL XTT in RPMI-1640 containing no additives with 6

μg/mL of PMS. The plates were then incubated for 4 hours at 37°C/5% CO_2 and read using a Spectramax spectrophotometer microplate reader at a wavelength of 450 nm and reference wavelength of 650 nm. Microsoft Excel 2010 in combination with XLfit4 were used to analyze and graph the data. EC_{25}, EC_{50} and EC_{95} (25%, 50% and 95% inhibition of virus replication), TC_{25}, TC_{50} and TC_{95} (25%, 50%, and 95% reduction in cell viability) and a therapeutic index (TI, TC_{25}/EC_{25}, TC_{50}/EC_{50}, TC_{95}/EC_{95}) were calculated using linear regression analysis.

Infectious Cell Center Assay (ICCA)

CEM-SS cells at a density of 5 x 10^5 cells/mL were infected with a pre-determined titer of HIV-1_{IIIB} for one hour. Following the initial infection, the cells and virus were transferred to a 25 cm^2 tissue culture (Corning) flask containing 5 mL of complete RPMI-1640. Twenty-four (24) hours post-infection, 5 mL of additional complete RPMI-1640 was added to the flask. Compound addition to the cultures was dependent on the variables being tested. Fixed concentrations of compound were added to cultures either prior to infection, immediately post infection, or at increasing time after infection. Cells were treated with compound for 12 consecutive days and the number of HIV-infected cells were quantified daily using the syncytium forming unit (SFU) assay (described below). Cell viability and total cell counts were determined using trypan blue dye exclusion. Treated and virus control (untreated) cells at a density of 3 x 10^5 cells/mL were washed two times with RPMI-1640 containing no additives to remove residual compound, and were added to uninfected CEM-SS cells at a density of 1 x 10^5 cells/well in a 96-well plate flat-bottomed plate (Corning). Forty-eight (48) hours post co-cultivation, the number of syncytia were quantified (1 syncytium = 1 infected cell) and

the number of infected cells in the cultures were calculated. Treated cells in the ICCA cultures were passaged by dilution every three days, or if the density was greater than 1.0 x 10^6 cells/mL in the cultures. After 12 days, all cultures remaining negative for syncytium formation were washed three times with RPMI-1640 with no additives. These cultures were monitored daily for CPE in the flasks, and cells and virus containing supernatant samples were taken once weekly. The ICCA was also performed under conditions that would mimic the environment under which a microbicide product might act, including: 1) Delayed compound addition, where a fixed concentration of compound was not added to the cultures post infection with HIV-1_{IIIB}. Infected CEM-SS cell were incubated for pre-determined time points (hours to days post infection) before compound was added to the cultures; 2) Pre-treatment of compound where CEM-SS cells were pretreated with fixed concentrations of compound prior to infection with HIV-1_{IIIB}. In this experiment, CEM-SS cells were incubated for pre-determined amounts of time with compound and then infected with HIV-1_{IIIB}. No additional compound was added to the cultures post infection; 3) Culture conditions were altered to remove compound from the infected CEM-SS cell cultures after pre-determined time points. This was performed by washing the infected CEM-SS cells 3 times with RPMI-1640 containing no additives and adding them back to complete RPMI-1640 with no additional compound; 4) Culture conditions were altered by dilution of compound over time to mimic compound pharmacokinetics in the vagina. Infected CEM-SS cells were cultured with pre-determined concentrations of the compound and at pre-determined time points, and fresh complete RPMI-1640 was added to the cultures to dilute the concentration of compound in the cultures.

Syncytium Forming Unit (SFU)Assay

To initiate the syncytium forming unit assay, cells from the ICCA cultures at a cell density of 3 x 10^5 cells/mL were removed from each ICCA culture and were washed with RPMI-1640 containing no additives and diluted logarithmically in complete RPMI-1640. Diluted cells, ranging from 1 x 10^5 cells/mL to 1 x 10^1 cells/mL were added to a 96-well, U-bottomed microtiter plate at a volume of 100 µL/well. Uninfected, CEM-SS cells were added to the plate at a density of 1 x 10^5 cells/well in a volume of 100 µL/well in all wells. Infected and uninfected cells were added to the plate in triplicate. A cell control was evaluated in parallel and included 100 µL of uninfected CEM-SS cells and assay media in place of the infected cells. Infected and uninfected cells were incubated in a humidified incubator at 37°C/5% CO_2 for 48 hours. Post incubation, syncytia were visually counted in each well of the plate and the number was recorded. If the total number of syncytia present was above 100, a > 100 was recorded. The numbers of syncytia for each density of infected cells was averaged and divided by the total number of infected cells present in the wells. This number was then multiplied by 100 to yield a percentage of infected cells in the culture, assuming 1 syncytium = 1 infected cell. Percentages of infected cells were graphed using an Excel template. This method was repeated every 24 or 48 hours post initial infection on cultures with viabilities above 20%.

DNA Extraction

Cellular DNA was extracted from virus infected cells using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) following the manufacturers recommended protocol. Briefly, the samples were lysed using proteinase K and the lysate was

transferred to the DNeasy Mini spin column and centrifuged. The DNA bound to the DNeasy membrane while any other contaminants were washed away using a series of buffers. The DNA was then eluted into water in a volume of 200 µL and was analyzed using polymerase chain reaction (PCR).

Amplification of Integrated Viral DNA Using PCR

Extracted DNA obtained from CEM-SS cells was amplified by PCR, using Taq Pro Complete (Biorad, Hercules, CA) or FideliTaq PCR Master Mix (Affymetrix). The primers used to amplify the RT enzyme were RT2551F (5'-GTTGACTCATGGTTGC-3') and BSP1286R (5'-GCCTCTGTTAATTCTTTTACATCA-3'). For each sample the following was added: 12.5 µL Taq Pro Complete or FideliTaq, 1 µL forward primer (RT2551F), 1 µL reverse primer (BSP1286R) and 5.5 µL dH_2O. Five microliters (5 µL) of extracted DNA was added from each sample to be tested, bringing the total volume to 25 µL. The PCR cycling conditions were a single cycle at 94°C for 5 minutes, followed by 34 cycles of 94°C for 30 seconds, 48°C for 30 seconds, 72°C for 2 minutes and a single cycle at 72°C for 10 minutes, and a hold at 4°C. Five microliters (5 µL) of the PCR product was added to 2 µL of 5X loading dye and then resolved and examined on a 0.8% agarose TAE gel stained with 0.2 µg/mL ethidium bromide to view the bands. A band on the gel indicated the presence of HIV viral DNA in the cell pellet extracts. A 10 kB HyLadder (Harvard Appartus, previously Denville Scientific, Holliston, MA) was used confirm amplification of the correct product.

RESULTS

Determination of HIV-1_{IIIB} Titer to Be Used in the Infectious Cell Center Assay (ICCA)

To determine the effect of varying concentrations of HIV-1_{IIIB} on the kinetics of infection in the ICCA in the absence of compound and to determine the optimal duration of the SFU assay, a detailed biological evaluation of the infection was performed daily following infection with varying multiplicities of infection (MOI) of virus. This evaluation included monitoring cell density, cell viability, syncytium forming units and RT. CEM-SS cells were infected with varying concentrations of HIV-1_{IIIB} at dilutions of stock virus of 1:50, 1:150 or 1:450. Cell density and cell viability were monitored each day for 9 days. It was determined that the cells in those cultures infected with a 1:50 or 1:150 dilution of virus had a peak cell density at day 5, and cells in the culture infected with a 1:450 dilution of virus had a peak cell density on day 7. Fifty percent (50%) cell viability occurred on day 8 for cells infected with a 1:50 dilution of virus and after day 9 in cells infected with a 1:150 and 1:450 dilution of virus, although viability was clearly decreasing at day 9. These data are presented in Figure 3 and Figure 4.

The cells from each infected culture on each day of infection (up to 8 days) were co-cultured with uninfected CEM-SS cells for up to 72 hours to determine the optimal timeframe for counting syncytia in the cultures. It was assumed that 1 syncytium resulted from 1 infected cell and therefore the percentage of infected cells present in the cultures could be quantitatively determined. Following 24 hours of co-cultivation, it was determined that approximately 1% to 5% of the cells in the cultures were infected. At 48 hours following infection, 10% to 50% of the cells in the cultures were infected, with peak infection on day 5 in the 1:50 culture and day 7 in the 1:150 and 1:450 culture. The

percentage of infected cells as measured by SFU assay at 72 hours and 48-hours of culture were similar, except peak infection occurred on day 5 for the 1:50 culture, day 7 for the 1:150 culture and day 8 for the 1:450 culture. These data are presented in Figure 5A-C.

Peak virus production was evaluated by measuring the amount of RT in the supernatant from the infected cell cultures over 9 days by RT assay. The RT assay measures incorporation of a radioactive precursor into elongating strands and the activity defined is directly proportional to the amount of RT in the cellular supernatant. Peak RT was observed on day 7 for cultures infected with a 1:50 dilution virus and on day 8 for cultures infected with a 1:150 dilution and 1:450 dilution of virus. These data are presented in Figure 6.

The data obtained indicate that the optimal dilution of virus to be used in the ICCA was a 1:50 dilution of stock virus, which would yield peak cell density and viability on day 5 and peak production of virus on day 6. Additionally, it was determined that co-cultivation of infected cells from the culture incubated with uninfected target cells for 48 hours would be the best timepoint for enumerating syncytia in order to determine the percentage of infected cells in the culture. The results demonstrate that the number of infected cells, cell viability and RT were all correlated to the timing of infection, as would be hypothesized.

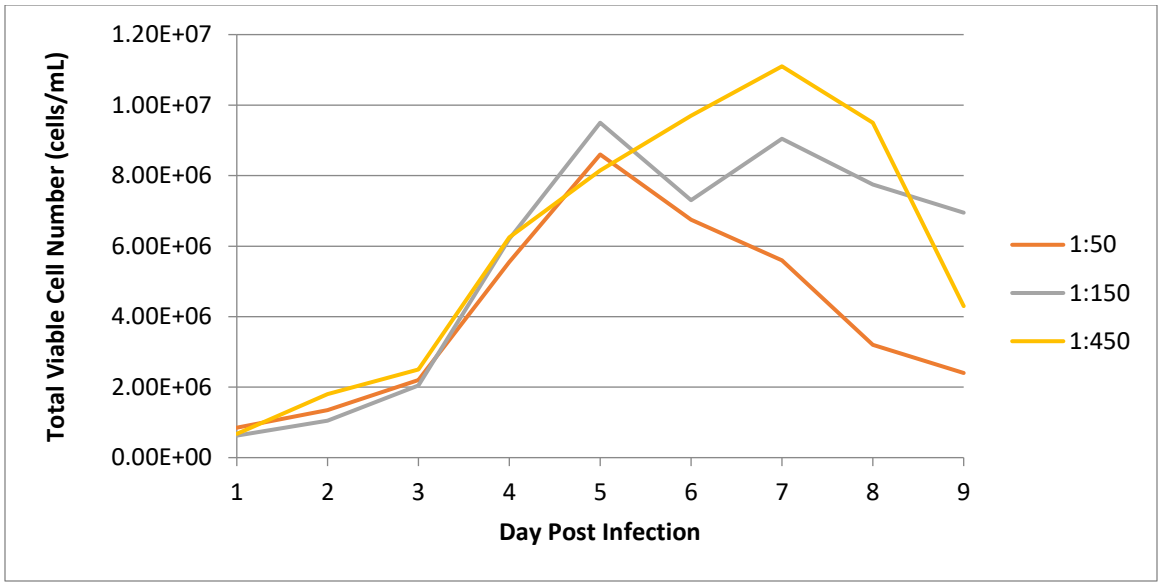


Figure 3. Viable cell density of CEM-SS cells infected with HIV-1_{IIIB} at varying MOIs. Cell density at each MOI are similar until after day 5. Cells infected with a 1:50 dilution of virus decrease in cell number whereas cells infected with a 1:150 or 1:450 dilution of virus maintained peak cell density until day 7 before decreasing.

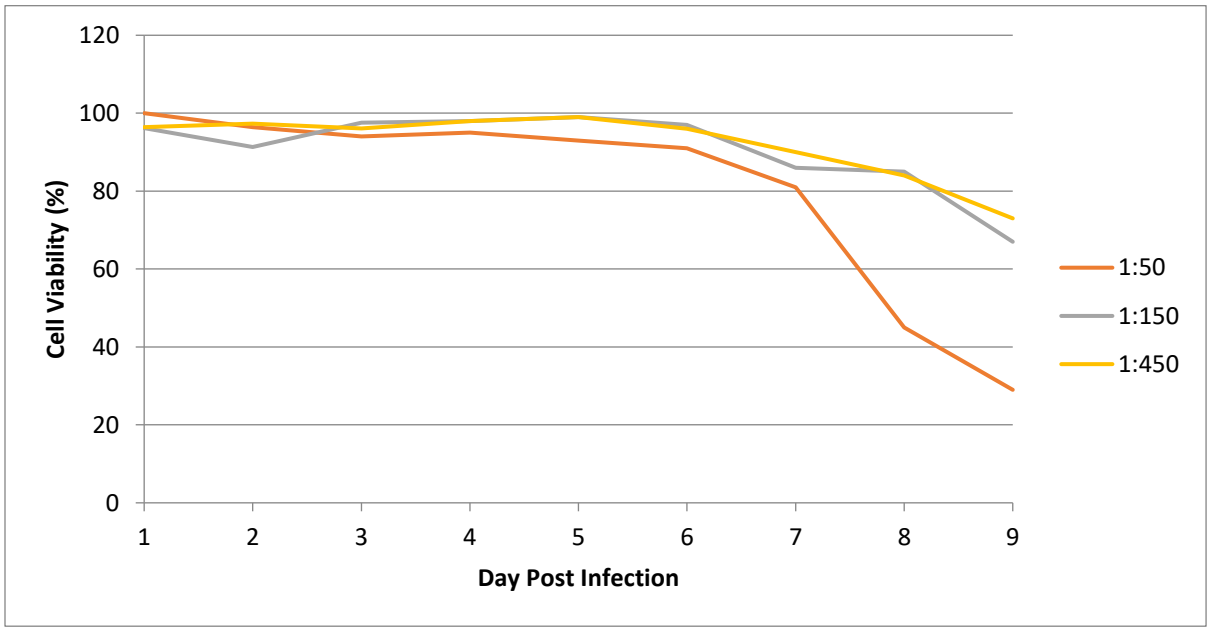


Figure 4: Cell viability of CEM-SS cells infected with HIV-1_{IIIB} at varying MOIs. Viabilities of each culture at each virus dilution are similar until day 8 and day 9 of the assay. On these days the culture infected with a 1: 50 dilution of virus resulted in a decrease in viability before the cultures infected with a 1:150 and 1:450 dilution of virus, which was expected due to the increased volume of virus present in the culture.

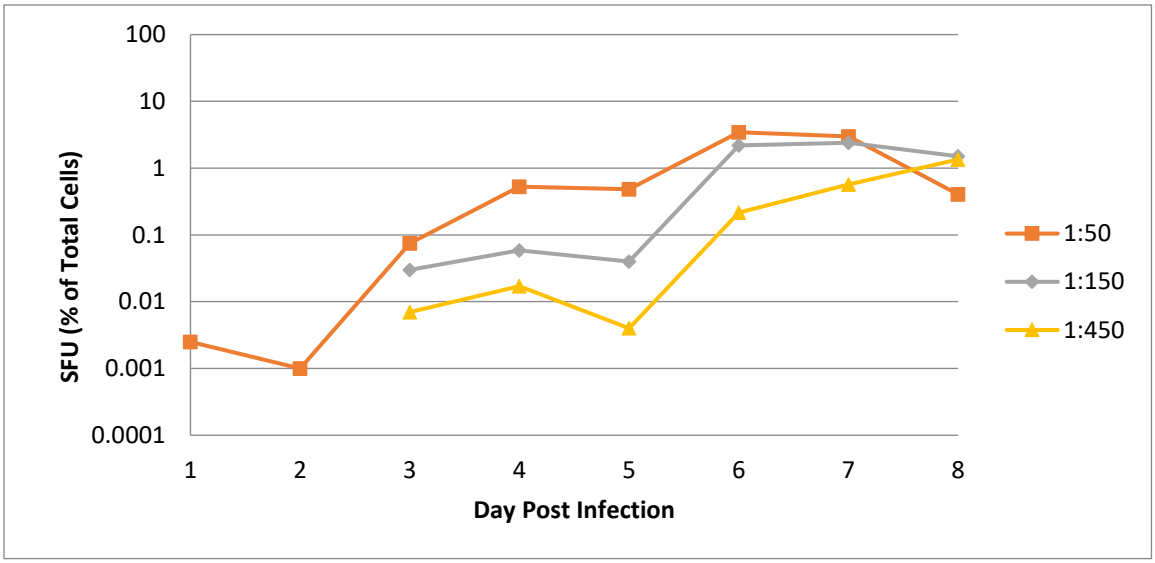


Figure 5A. Percentage of Infected Cells at 24 Hours Following Co-Cultivation with Uninfected CEM-SS Cells Cultured for 8 Days. The cultures infected with a 1:50 dilution and 1:150 dilution of HIV-1_{IIIB} had peak infection on day 6. In the culture infected with a 1:450 dilution of HIV-1_{IIIB} peak infection was observed on day 8.

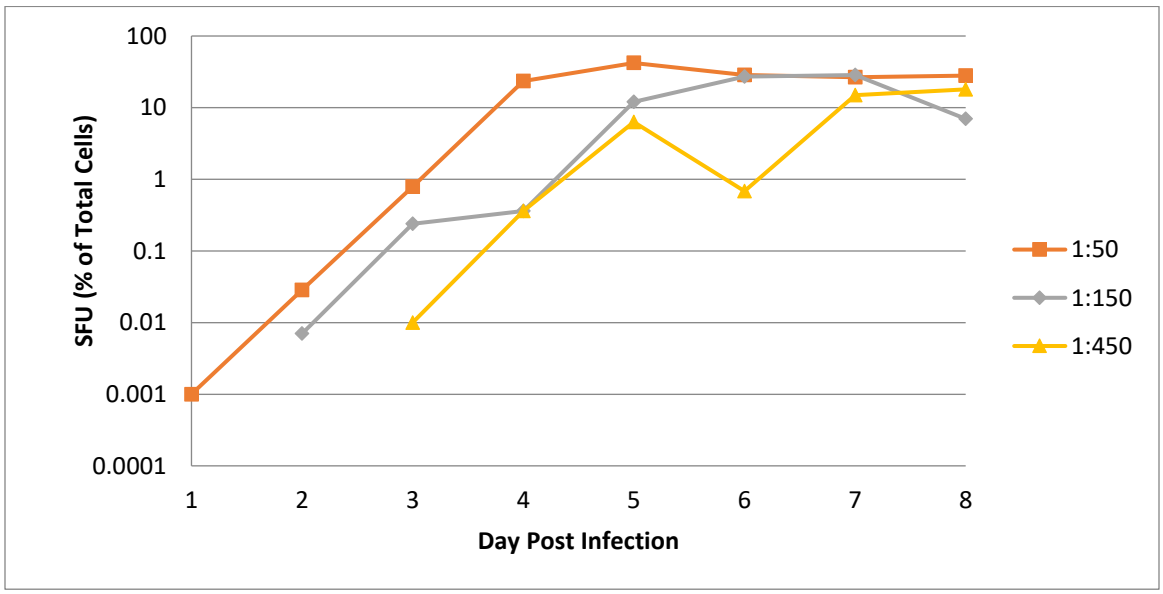


Figure 5B. Percentage of Infected Cells at 48 Hours Following Co-Cultivation with Uninfected CEM-SS Cells Cultured for 8 Days. The culture infected with a 1:50 dilution has peak infection on day 5. The culture infected with a 1:150 dilution of HIV-1_{IIIB} had peak infection on day 6. In the culture infected with a 1:450 dilution of HIV-1_{IIIB} peak infection was observed on day 8.

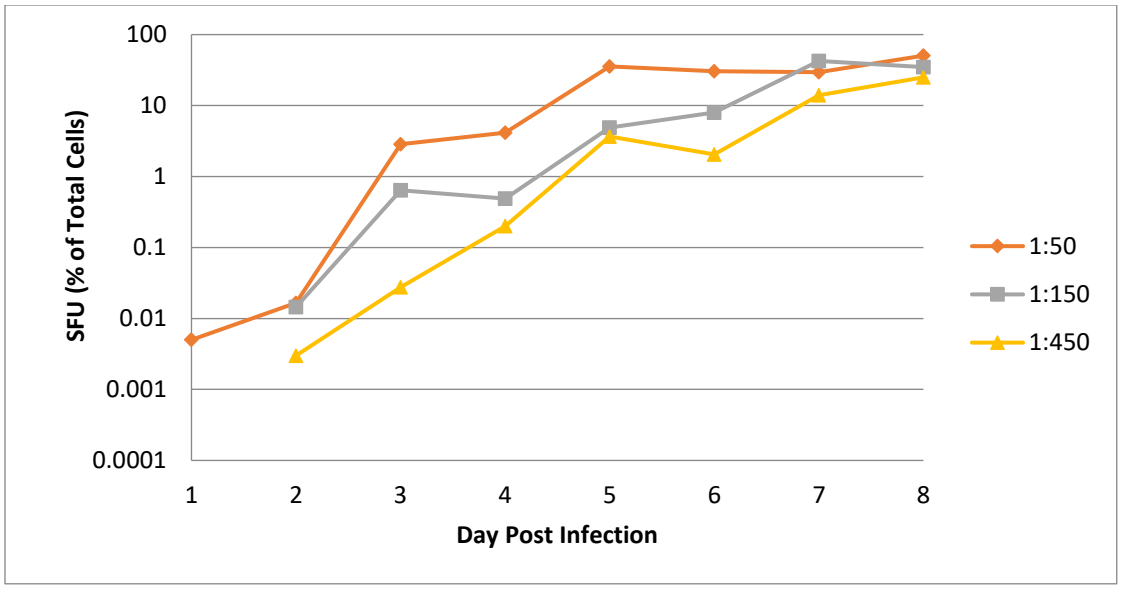


Figure 5C. Percentage of Infected Cells at 72 Hours Following Co-Cultivation with Uninfected CEM-SS Cells Cultured for 8 Days. The culture infected with a 1:50 dilution has peak infection on day 5. The culture infected with a 1:150 dilution of HIV-1_{IIIB} had peak infection on day 7. In the culture infected with a 1:450 dilution of HIV-1_{IIIB} peak infection was observed on day 8.

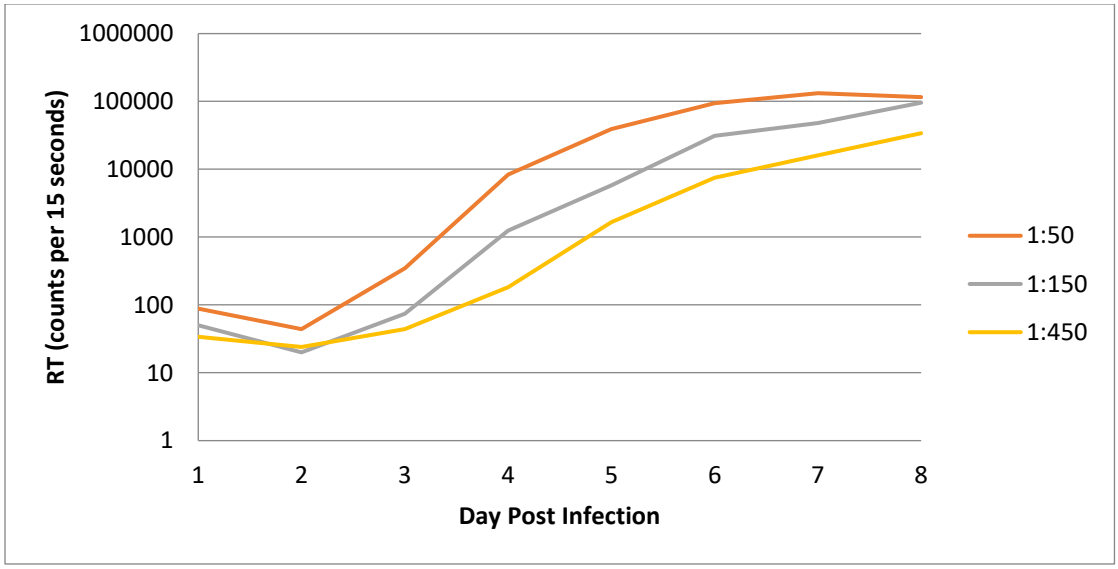


Figure 6. RT in the culture supernatant measured in the HIV-1_{IIIB} ICCA cultures infected at three MOIs. The results show a distinct difference between each of the three dilutions, with the higher concentration of virus having the highest counts per 15 seconds and an earlier peak day compared to the lower MOI cultures.

Comparison of HIV-1_{IIIB} and HIV-1_{RF} in the ICCA

To determine if comparable results were obtained in the ICCA with different laboratory strains of HIV-1, the ICCA was performed with HIV-1_{IIIB} and HIV-1_{RF}. In addition, the sensitivity of the assay was evaluated by determining if a two-fold differences in input virus could be distinguished in the ICCA. It was important to determine if noticeable shifts in cell densities, cell viabilities and RT could be observed when varying amounts of virus was added to the ICCA. A detailed biological evaluation of these infected cultures was taken daily which included monitoring cell density, cell viability and syncytium formation.

In this comparative assay, the virus inoculums were diluted from stock virus at dilutions of 1:25, 1:50 and 1:100. Cultures infected with HIV-1_{IIIB} had varying peak days of cell density, with the culture infected with a 1:25 dilution of virus peaking on day 4, the culture infected with a 1:50 dilution of virus peaking on day 5 and the 1:100 dilution of virus peaking on day 6. These data are presented in Figure 7A. Each HIV-1_{RF} infected culture had a peak cell density on day 5 but a different total viable cell density. The highest viable cell density was observed in cultures with higher dilutions of virus (i.e. less virus in culture). These data are presented in Figure 7B.

A decrease to 50% cell viability occurred at day 8 for the cultures infected with a 1:25 dilution of HIV-1_{IIIB} and on day 9 for the cultures infected with a 1:50 and 1:100 dilution of HIV-1_{IIIB}. In the cultures infected with HIV-1_{RF} a decrease to 50% cell viability was observed on day 7 for the cultures infected with a 1:25 dilution of HIV-1_{RF}, day 8 for the cultures infected with a 1:50 dilution of HIV-1_{RF} and day 9 for the cultures infected with a 1:100 dilution of HIV-1_{RF}. Data are presented in Figures 8A and 8B.

Quantification of RT in these cultures demonstrated that the cultures infected with a 1:25 dilution of HIV-1_{IIIB} had peak RT on day 7 and the cultures infected with a 1:50 and 1:100 dilution of HIV-1_{IIIB} had peak RT on day 10. The cultures infected with a 1:25 dilution of HIV-1_{RF} had peak RT on day 5 and the cultures infected with a 1:50 and 1:100 dilution of HIV-1_{RF} had peak RT on day 6. In both cultures, those that were infected with more concentrated virus corresponded with higher levels of RT production in the cell free supernatants. These data are presented in Figure 9A and B.

When co-cultured with uninfected CEM-SS cells for 48 hours, the culture infected with a 1:25 dilution of HIV-1_{IIIB} was 80 to 90% infected by day 6. The culture infected with a 1:50 dilution of HIV-1_{IIIB} reached peak infection with greater than 50% of the cells in the culture infected on day 6, and the culture infected with a 1:100 dilution of HIV-1_{IIIB} had peak infection on day 10 with 50% of the cells in the culture infected. The culture infected with a 1:25 dilution of HIV-1_{RF} had peak infection on day 6 with 100% of the culture infected. The culture infected with a 1:50 dilution of HIV-1_{RF} had peak infection on day 4, but with only 50% of the cells in the culture infected. The culture infected with a 1:100 dilution of HIV-1_{RF} had peak infection on day 7 with 50% of the cells in the culture infected. Data are presented in Figure 10A and B.

These data suggest that the optimal dilution of HIV-1_{IIIB} to be used in the ICCA was a 1:50 dilution, which is identical to the optimal inoculum determined in the initial analysis. These data would also suggest that HIV-1_{RF} could be used in the ICCA at a 1:50 dilution, which would yield peak cell densities on day 5 and peak production of virus on day 6. It was additionally determined that the ICCA can distinguish between a two-fold dilution of virus in all biological evaluations that were performed.

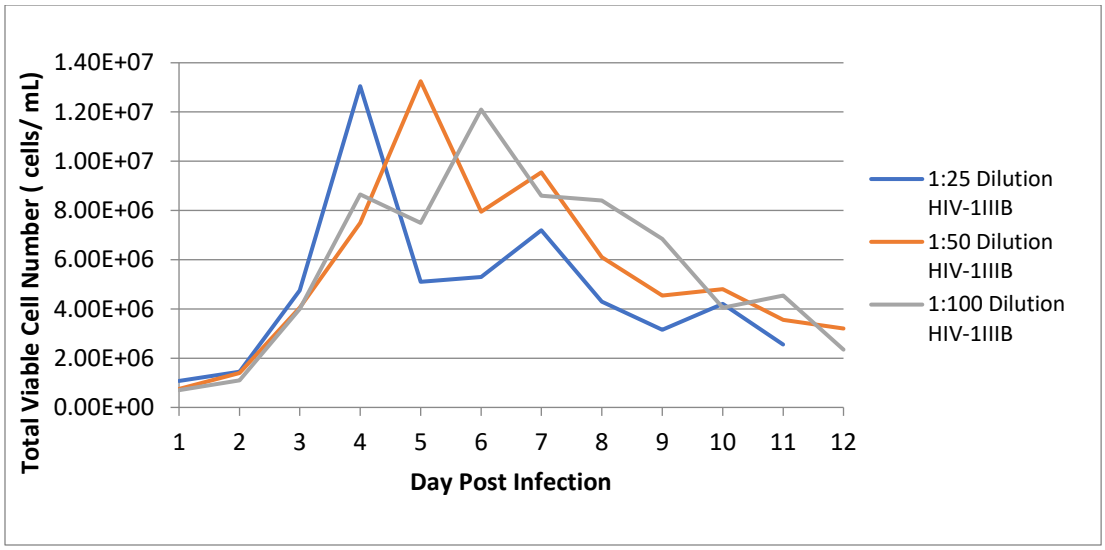


Figure 7A. Total viable cell density in HIV-1_{IIIB} infected cultures. The culture infected with a 1:25 dilution of HIV-1_{IIIB} reached peak cell density at day 4. The cultures infected with a 1:50 dilution and 1:100 dilution of HIV-1_{IIIB} reached peak density on day 5 and day 6, respectively. Peak total viable cell density was similar for each culture with each dilution of HIV-1_{IIB}.

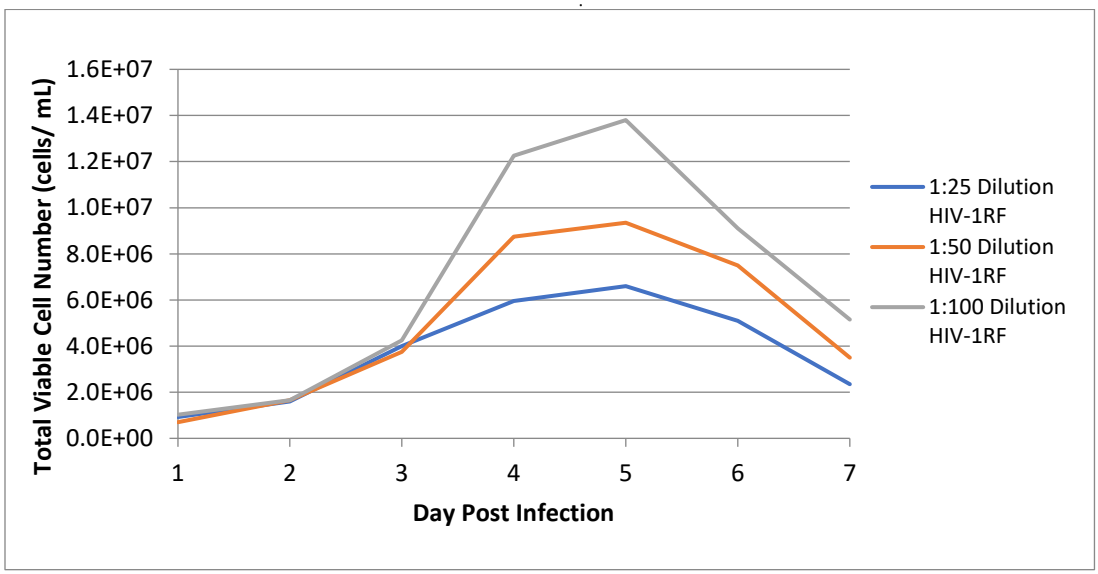


Figure 7B. Total viable cell density in HIV-1_{RF} infected cultures. All cultures reached peak cell density at day 5. Lower cell density was proportional to the amount of virus in the culture i.e. the cultures with more virus (1:25) had lower cell density.

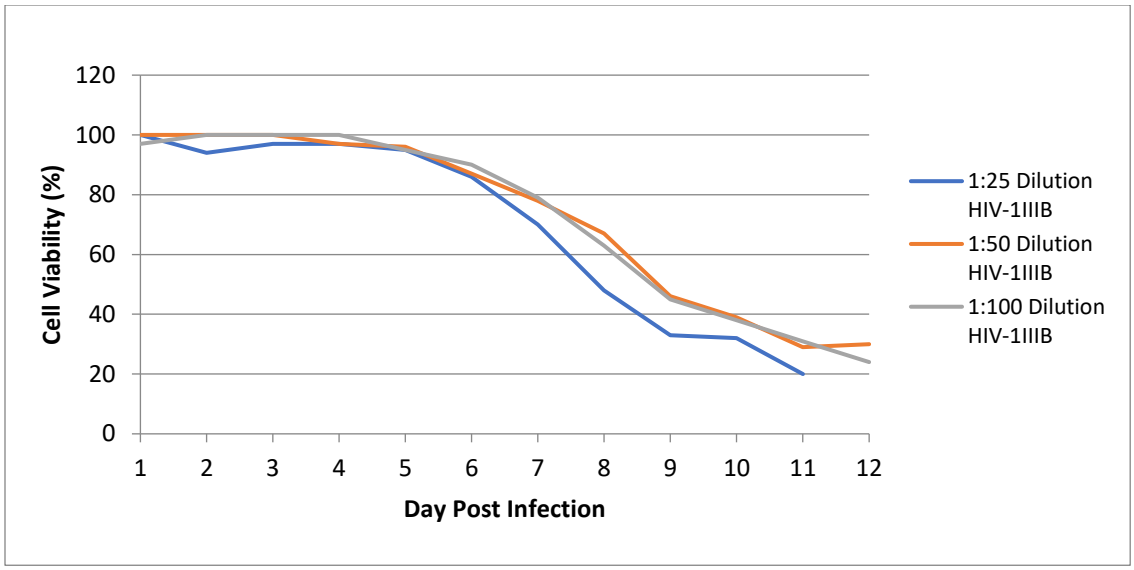


Figure 8A. Cell viability in HIV-1_{IIIB} infected cultures. Viability decreases in all the infected cultures at day 5 or 6. By day 8 the culture infected with a 1:25 dilution of HIV-1_{IIIB} has less than 50% cell viability and the cultures infected with a 1:50 dilution and 1:100 dilution of virus reaches less than 50% viability by day 9.

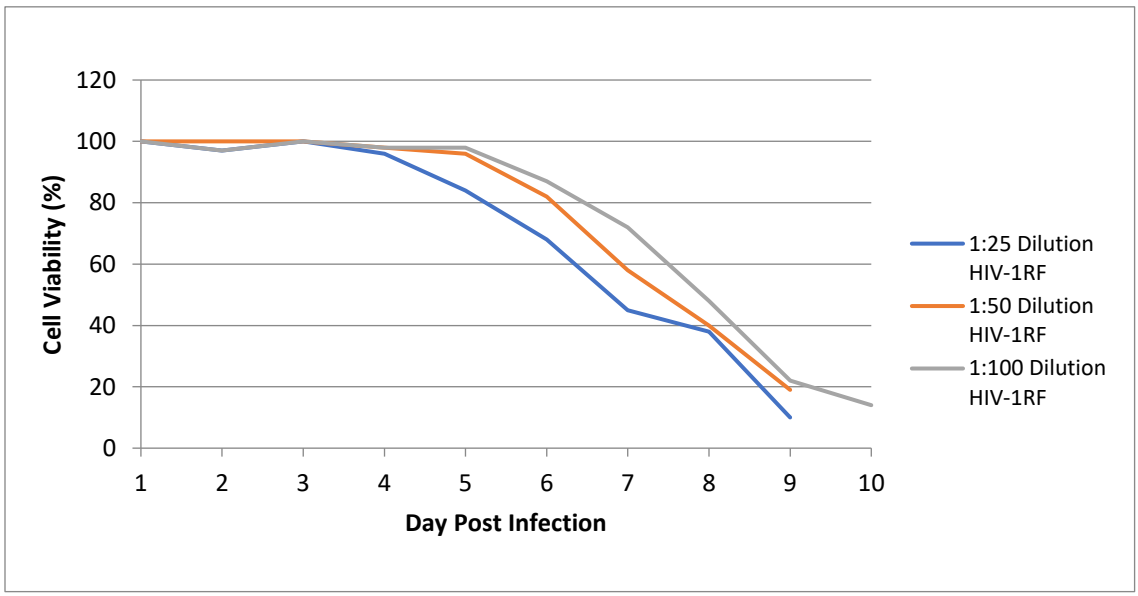


Figure 8B. Cell viability in HIV-1_{RF} infected cultures. In the culture infected with a 1:25 dilution of HIV-1_{RF} cell viability begins to decrease at day 4 and reaches less than 50% viability at day 7. The culture infected with a 1:50 dilution of virus decreases to less than 50% viability by day 8, while the culture infected with a 1:100 dilution of virus culture decreases to a viability of less than 50% by day 9.

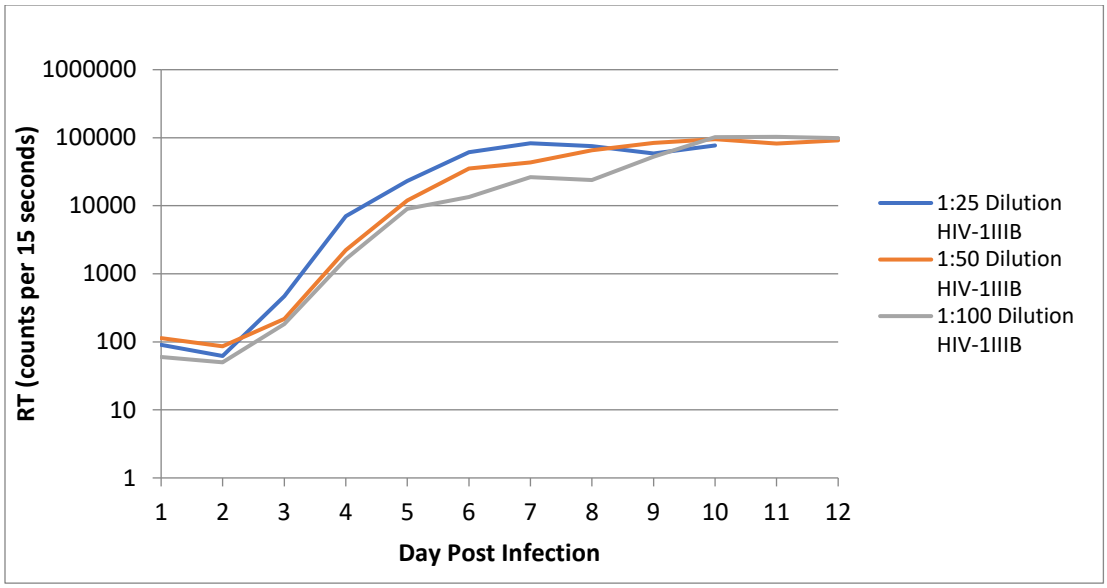


Figure 9A. RT in HIV-1_{IIIB} infected cultures. The peak RT for the cultures infected with a 1:25 dilution of HIV-1_{IIIB} was on day 7 and the peak RT for the cultures infected with a 1:50 and 1:100 dilution of HIV-1_{IIIB} was on day 10. The culture infected with a 1:25 dilution of HIV-1_{IIIB} had higher RT than the cultures infected with a 1:50 and 1:100 dilution of HIV-1_{IIIB}.

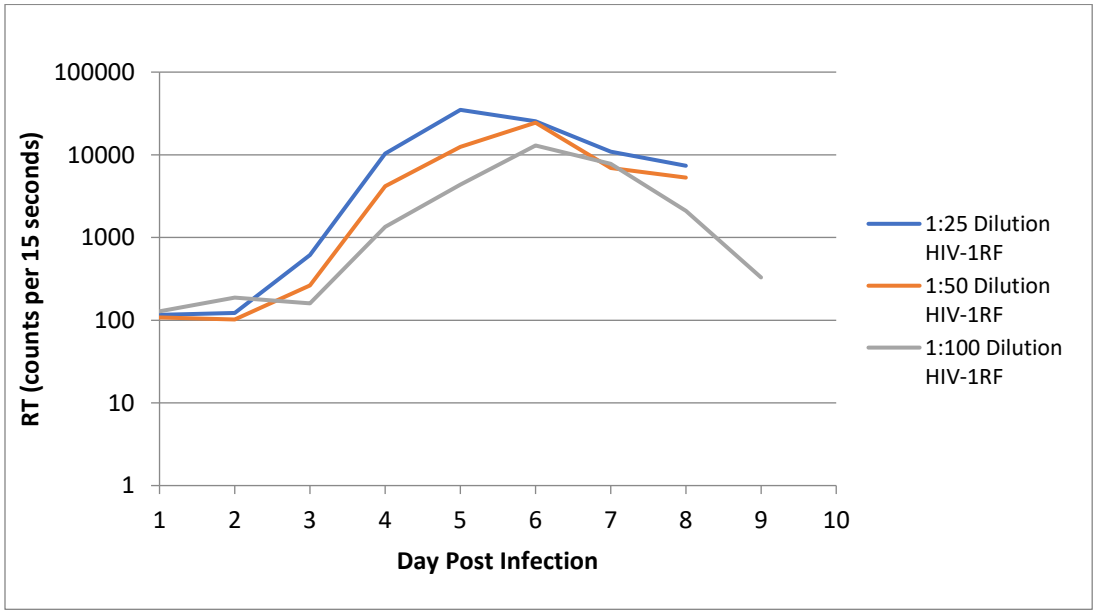


Figure 9B. RT in HIV-1_{RF} infected cultures. The cultures infected with a 1:25 dilution of HIV-1_{RF} had peak RT on day 5 and the cultures infected with a the 1:50 and 1:100 dilution of HIV-1_{RF} had peak RT on day 6. Higher dilutions of virus (less virus in the cultures) had less RT.

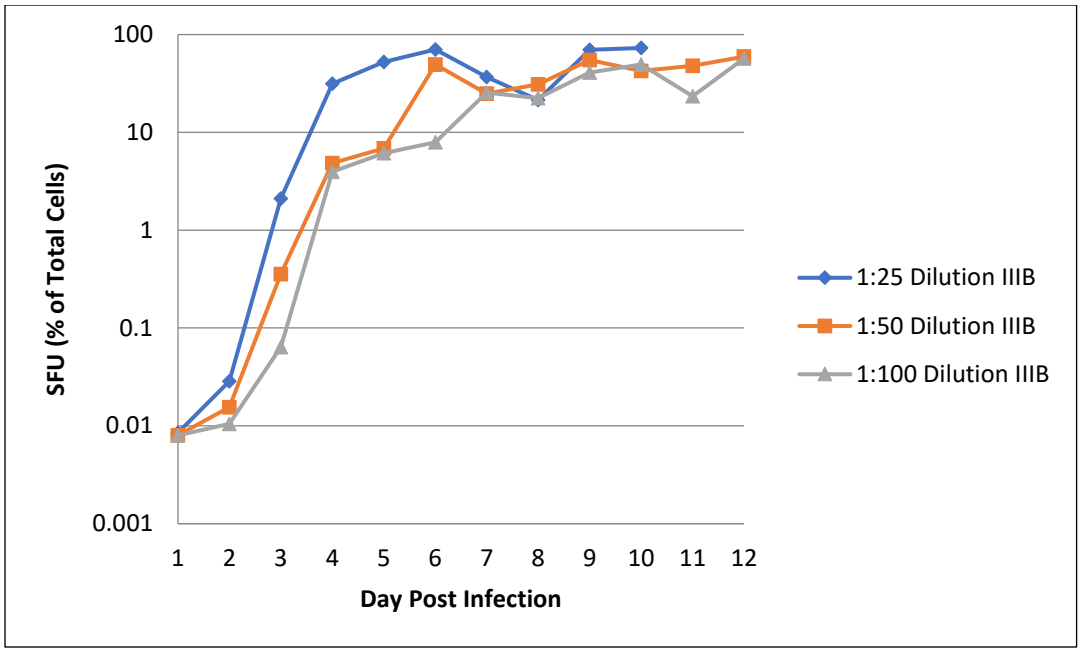


Figure 10A. Percentage of infected cells in HIV-1_{IIIB} infected cultures at 48 hours. The cultures infected with a 1:25 dilution and 1:50 dilution of HIV-1_{IIIB} had peak infection on day 6. In the culture infected with a 1:100 dilution of HIV-1_{IIIB} peak infection was observed on day 10.

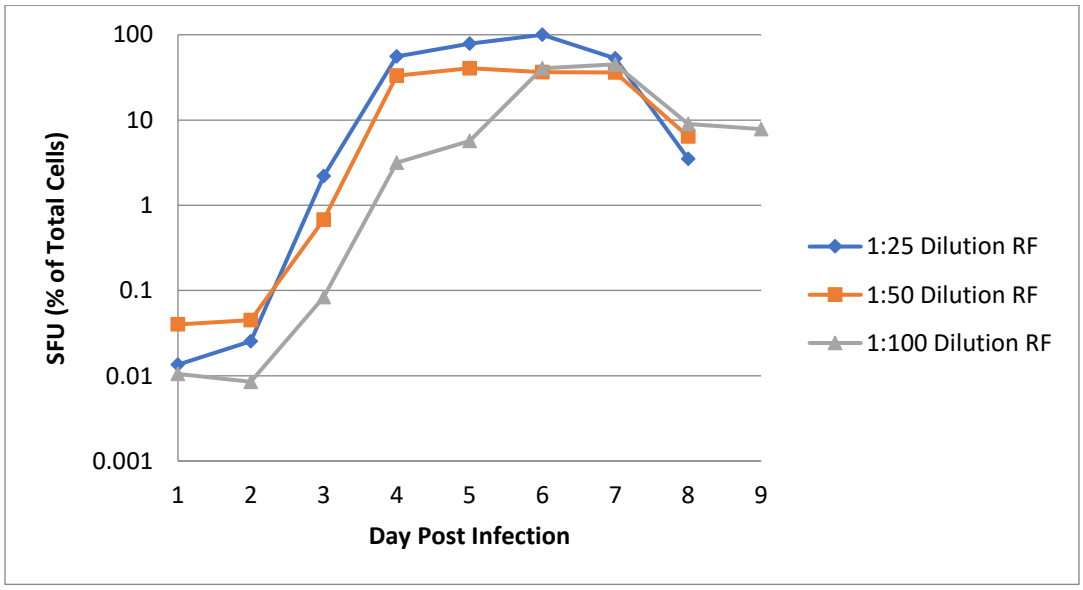


Figure 10B. Percentage of infected cells in HIV-1_{RF} infected cultures at 48 hours. The cultures infected with a 1:25 dilution of virus had peak infection on day 6. The culture infected with a 1:50 dilution of virus had peak infection on day 4. The culture infected with a 1:100 dilution of virus had peak infection on day 7.

Evaluation of HIV-1_{IIIB} Transmission When Evaluated in the ICCA at Defined Concentrations of IQP-0528

To determine the sterilizing concentration of IQP-0528 in the ICCA and to evaluate the effect of IQP-0528 on virus transmission in the ICCA, the ICCA was performed in the presence of a range of IQP-0528 concentrations chosen based on the published results defined in assays performed in a precursor MTSA assay (Watson *et al.* 2007). The ICCA was performed using the same method as described above, with a 1:50 dilution of stock HIV-1_{IIIB}. Concentrations of IQP-0528 used in this initial ICCA were 0.002 μM, 0.05 μM and 1 μM.

When evaluating the percentage of infected cells in the culture as measured by syncytium formation, the 0.002 μM treated culture had a peak infection on day 10 with greater than 50% of the cells in culture infected. The 0.05 μM and 1 μM treated culture remained below the limit of detection defined in the SFU assay which was 0.001% or 1 infected cell in 100,000. These data are presented in Figure 11A.

When evaluating virus replication by measuring RT, the 0.002 μM treated culture had peak RT on day 10. The 1 μM treated culture had peak RT on day 8 but remained near background levels of RT for the remainder of the assay. The 0.05 μM treated culture had RT comparable to background levels seen in uninfected CEM-SS cells. These data are presented in Figure 11B.

These results indicate that the addition of IQP-0528 to the cell culture directly impacts the transmission and replication of HIV-1_{IIIB}, with a sterilizing concentration ranging between 0.05 μM and 1 μM in the ICCA.

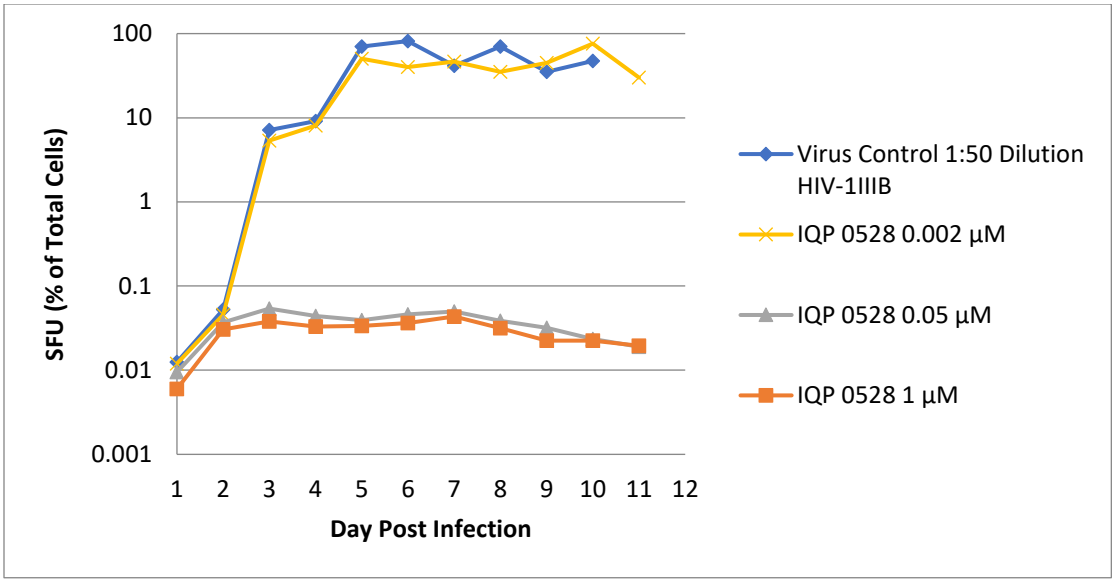


Figure 11A. Percentage of total cells infected in the ICCA 48 hours post infection with HIV-1_{IIIB} in the presence of varying concentrations of IQP-0528. The 0.002 µM treated culture had a peak infection on day 10. The 0.05 µM and 1 µM treated cultures remained below the limit of detection in the SFU assay.

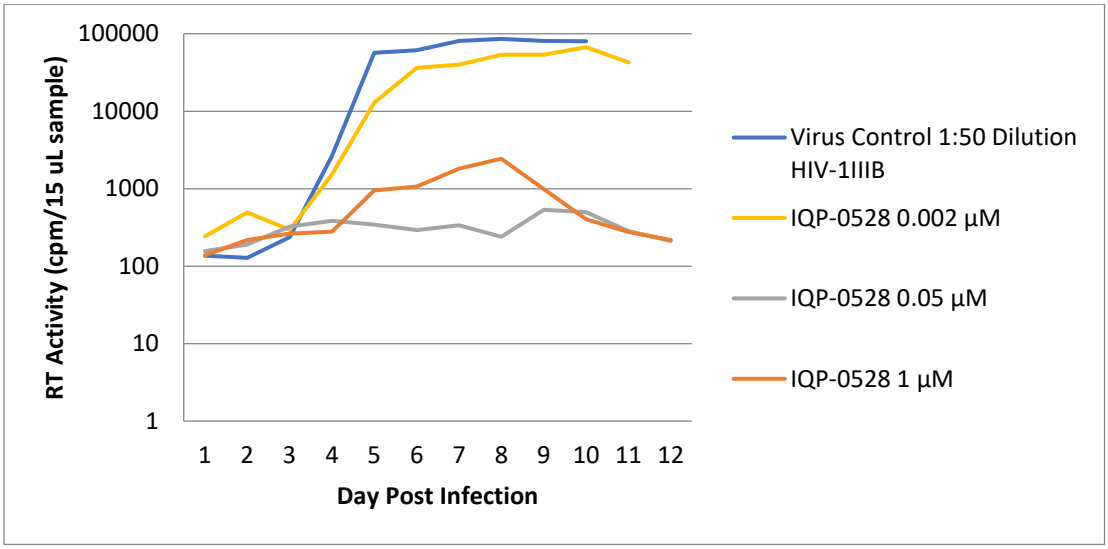


Figure 11B. RT observed in cell cultures infected with HIV-1_{IIIB} in the presence of IQP-0528 at varying concentrations. The 0.002 µM treated culture had peak RT on day10. The 0.05 µM treated culture had RT at background level from day 1 to day 12 of the infection Slightly higher than background RT was observed in the 1 µM treated culture from day 5 to day 9 and then levels returned to background levels from day 10 to day 12.

Confirmatory Evaluation of HIV-1_{IIIB} Virus Transmission When Evaluated in the ICCA at Defined Concentrations of IQP-0528

To confirm the sterilizing concentration of IQP-0528 defined in the initial ICCAs with HIV-1_{IIIB} and to determine the sensitivity of the ICCA, ICCAs were repeated at concentrations of IQP-0528 that bracketed those used in the previous experiment, including 0.001 μM, 0.01 μM, 0.1 μM and 1 μM

The first confirmatory assay was performed according to the ICCA method performed previously. Additionally, the cultures which remained negative for virus infection at day 12 were kept in the presence of IQP-0528 to determine if virus breakthrough would occur if treatment of the cultures continued longer than 12 days. If breakthrough eventually occurred, we also wanted to ascertain if the breakthrough virus remained wild type or if a resistant virus would begin to replicate. These cultures with continuous treatment were monitored until day 30 of the assay. In a second permutation of the original ICCA methodology, cells from the cultures were also added to new culture flasks in the absence of IQP-0528. Cells from these cultures were washed at days 8, 13 and 16 to remove residual IQP-0528 and were cultured until day 48 of the ICCA. Compound removal at these days was performed to determine if there is a threshold of time at which IQP-0528 must be present in a culture to provide sterilization as well as to allow the outgrowth of any rare infected cell populations in the culture that persist in the presence of sub-sterilizing concentrations of IQP-0528.

The 0.001 μM treated culture in the ICCA resulted in virus breakthrough immediately, similar to the virus control, and peak infection occurred on day 8 of the ICCA with greater than 50% of the cells in culture infected. The 0.01 μM treated culture had a delay in the kinetics of infection and spread but reached peak infection on day 10

with 10% of the cells in culture infected. The 0.1 μM and 1 μM treated cultures remained below the limit of detection. These data are presented in Figure 12A.

Peak RT was observed on day 7 and day 10 in the cultures carried in the presence of 0.001 μM and 0.01 μM of IQP-0528, respectively. The culture with 0.1 μM IQP-0528 had a large increase in RT on day 2, but RT remained at background levels for the remainder of the assay. The culture carried in the presence of 1 μM IQP-0528 had RT levels comparable to background levels. These data are presented in Figure 12B.

Following the removal of IQP-0528 from the 0.1 μM and 1 μM treated cultures at day 8, 13 and 16, there was no CPE observed in the cultures through 30 days post compound removal. Unfortunately, tissue culture contamination occurred for both the 0.1 and 1 μM cultures with compounds removed on day 8 at day 25 and 34 (17- and 26-days post compound removal) respectively which prevented additional data from being recorded for this culture. However, both cultures remained negative for CPE until this point. The 0.1 and 1 μM treated cultures with compound removal on day 13 became contaminated on day 26 (13 days post compound removal). Again, both cultures remained negative for CPE until this point. Importantly for these evaluations, cultures with compound removal on day 16 remained contaminant free until the end of the experiment on day 30 and day 48 (32 days post compound removal). Both of these cultures remained negative for CPE. These data confirm that the sterilizing concentration of IQP-0528 is between 0.01 μM and 0.1 μM in the ICCA.

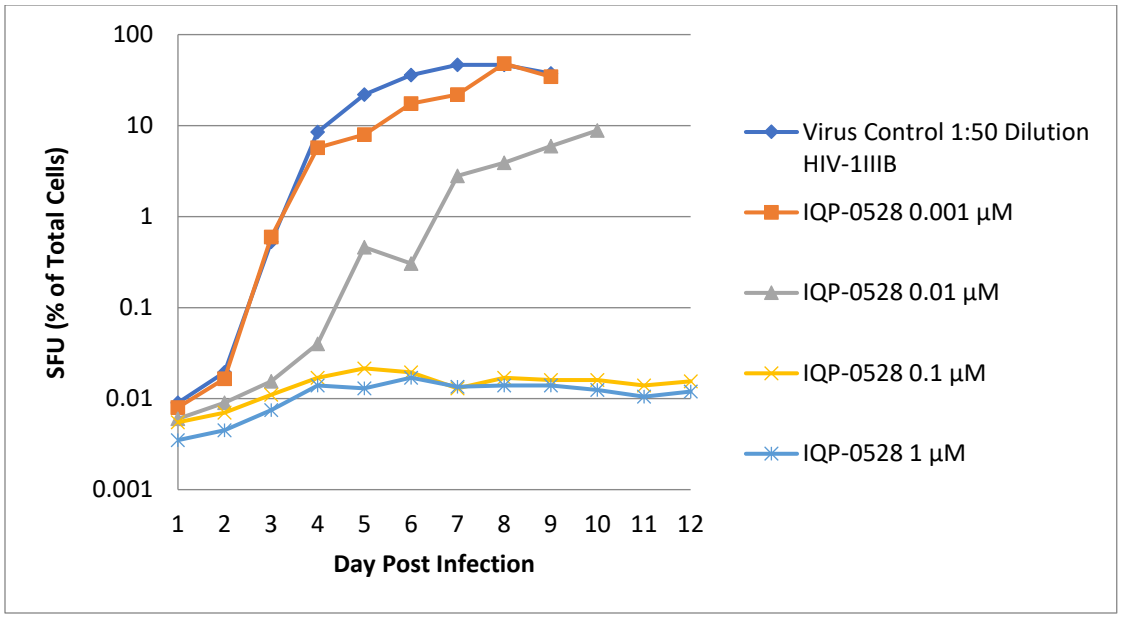


Figure 12A. Percentage of total cells infected in the ICCA with HIV-1_{IIIB} at varying concentrations of IQP-0528. Cultures treated with 0.001 µM and 0.01 µM had peak infection on day 8 and day 10, respectively. The 0.1 µM and 1 µM treated cultures had some syncytium observed between days 4 and 11 and remained slightly above the limit of detection.

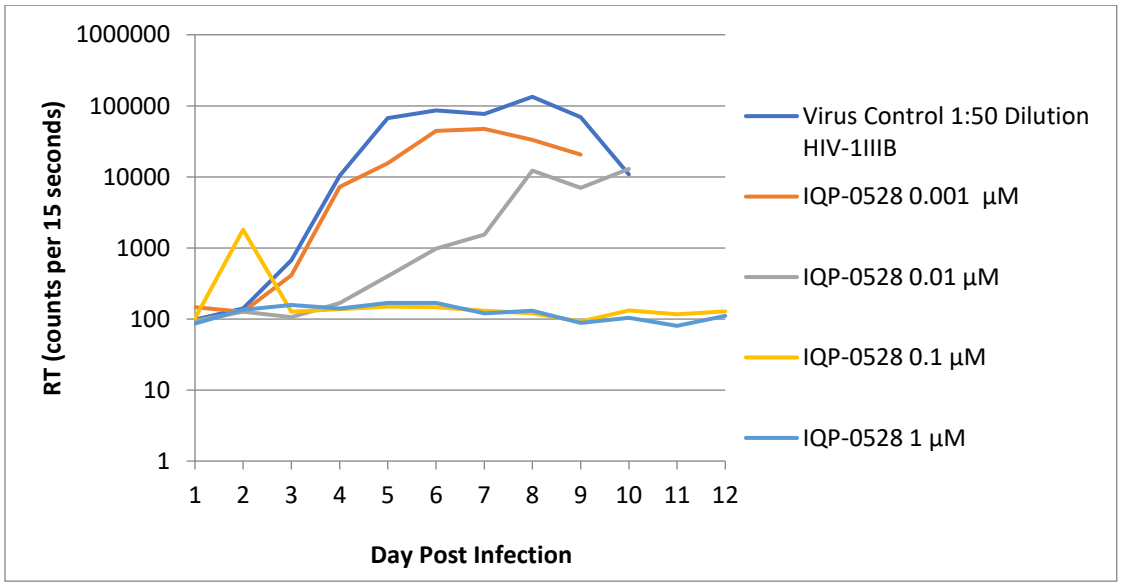


Figure 12B. RT in the ICCA with HIV-1_{IIIB} at varying concentrations of IQP-0528. The RT of the 0.001 µM IQP-0528 culture peaked on day 7. The 0.01 µM IQP-0528 culture had peak RT on day 10. The 0.1 µM treated culture had a slight increase in RT on day 2 but remained at background levels for the remainder of the assay. The 1 µM IQP-0528 culture had no RT above background levels

In addition to confirming the sterilizing concentration of IQP-0528, a second confirmatory ICCA was performed to determine if the ICCA can distinguish between a smaller range of IQP-0528 concentrations, including 0.0125 μM, 0.025 μM, 0.05 μM and 0.1 μM. Following day 12 of the ICCA, the cultures remaining negative for infection were washed three times to remove residual IQP-0528 and the cultures were continued in the absence of IQP-0528 to determine if rare infected subpopulations existed in these cultures. Each culture was monitored for the appearance of RT in the cell free supernatants and virus induced CPE o in the ICCA cultures, as well as infection based on SFU assay

In this ICCA, break-through of virus was observed in the 0.0125 μM and 0.025 μM treated cultures, with peak infection observed on day 8 in the 0.0125 μM culture, with 90% of the cells in the culture infected, and on day 10 in the 0.025 μM treated culture, with greater than 5% of the cells in culture infected. The 0.05 μM and 0.1 μM treated cultures remained below the limit of detection in the SFU assay until day 12 when the 0.05 μM treated culture increased above the limit of detection. These data are presented in Figure 13A.

Peak RT occurred on day 9 in the 0.0125 μM IQP-0528 treated culture and on day 11 in the 0.025 μM treated culture. The 0.05 μM and 0.1 μM treated cultures had no RT above background levels through the 12 days of the ICCA. These data are presented in Figure 13B.

IQP-0528 was removed from the 0.05 μM and 0.1 μM treated cultures on day 12 because no virus infection was observed up to that point. RT and CPE were monitored daily post compound removal. The 0.05 μM IQP-0528 culture had increasing CPE and

RT steadily after compound was removed on day 12, with peak RT occurring on day 6 post compound removal (day 18 of the assay). The 0.1 µM IQP-0528 treated culture had no increase in RT above background levels until day 8 post compound removal (day 20 of the assay), but the RT was still comparable to background activity. These data are shown in Figure 13C. These results would indicate that rare subpopulations existed in the 0.05 µM treated culture, indicating that this concentration of IQP-0528 was sub-optimal with regard to sterilization though it was highly active in the inhibition of virus replication and spread in the treated culture.

These data suggest that the sterilizing concentration of IQP-0528 is between 0.05 µM and 0.1 µM. These data also suggest that the ICCA can distinguish between 2-fold dilutions of compound and that the removal of compound is a critical step to accurately determine the concentration of a test compound that yields complete virus sterilization of the cultures.

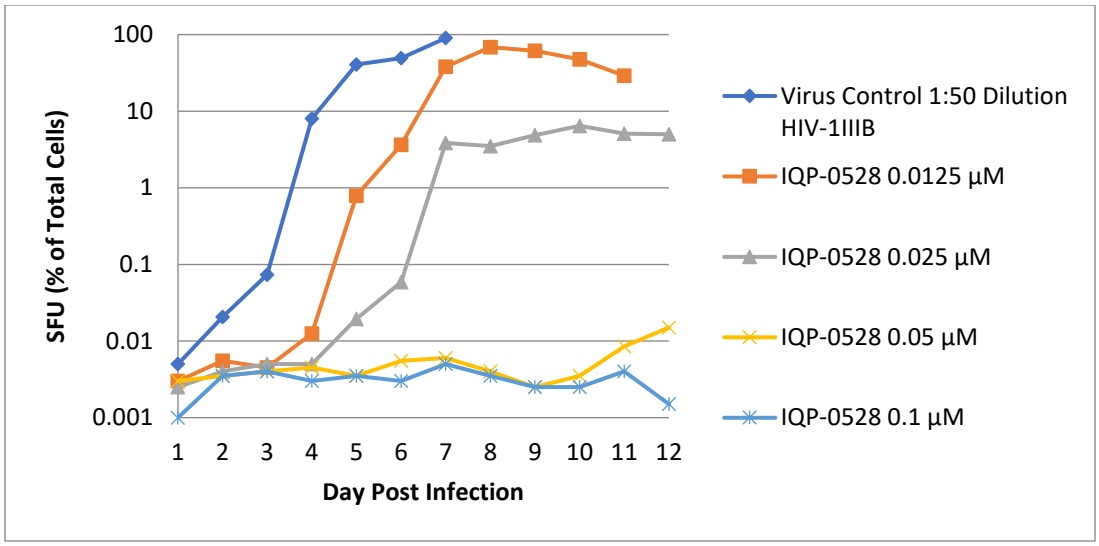


Figure 13A. Percentage of infected cells in ICCA cultures using a narrowed range of IQP-0528 concentrations- Confirmatory evaluation. The 0.0125 µM and 0.025 µM treated cultures had peak infection on day 8 and day 10, respectively. One SFU count observed on day 12 was above the lower limit of detection in the 0.05 µM treated culture. The 0.1 µM treated culture remained below the detectable limit of the SFU assay.

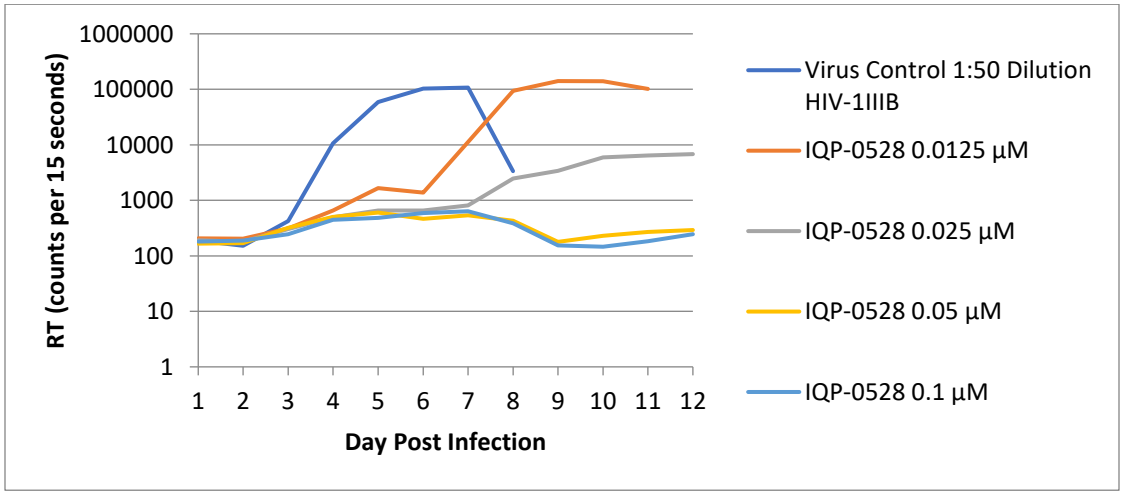


Figure 13B. RT in the ICCA with HIV-1_{IIIB} at a narrowed range of IQP-0528 concentrations- Confirmatory evaluation. The 0.0125 µM treated culture had peak RT on day 9 and the 0.025 µM treated culture had peak RT on day 11. The 0.05 µM and 0.1 µM treated cultures remained at background RT levels.

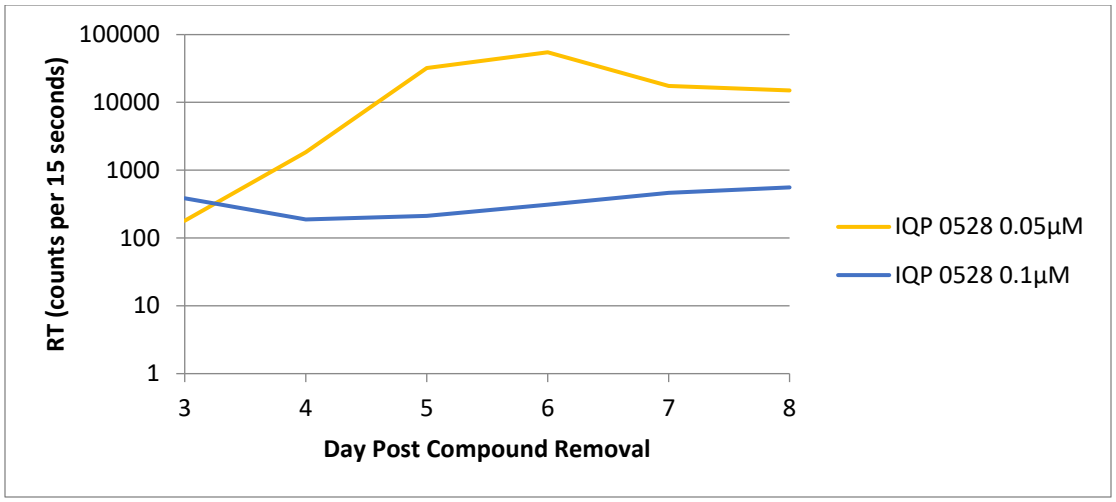


Figure 13C. RT in cultures following compound removal at day 12 in the ICCA. The 0.05 µM treated culture had increasing RT starting 3 days post compound removal, with peak RT on day 6 post compound removal. The 0.1 µM IQP-0528 culture remained at background RT levels.

Evaluation of HIV-1_{NL4-3} Transmission When Evaluated in the ICCA at Defined Concentrations of IQP-0528

To determine the effect of IQP-0528 on the transmission of HIV-1_{NL4-3}, two ICCAs were performed using the same methodology as described previously, using a 1:2 dilution of HIV-1_{NL4-3}. Concentrations of IQP-0528 were selected based of the results observed in the ICCAs performed with HIV-1_{IIIB} and included 0.01 μM, 0.05 μM, 0.1 μM, 0.2 μM and 0.5 μM IQP-0528.

The 0.01 μM treated culture had a peak infection on day 8 with greater than 10% of the cells in the culture infected. All other treated cultures remained below the limit of detection in the SFU assay. A small number of syncytia were observed only in the 0.05 μM treated culture on days 8, 10 and 12 and on day 9 in the 0.1 μM treated culture. Syncytia were not observed in these cultures on the other days of evaluation, and thus the cultures remained below the limit of detection. These data are presented in Figure 14A.

RT in the cell free supernatants was minimal for all cultures treated with IQP-0528 and the virus control culture. The virus control had peak RT on day 12. Increased RT was observed in the 0.01 μM treated culture in comparison to the other treated cultures from day 6 to day 12. All other cultures showed RT comparable to background levels. These data are presented in Figure 14B.

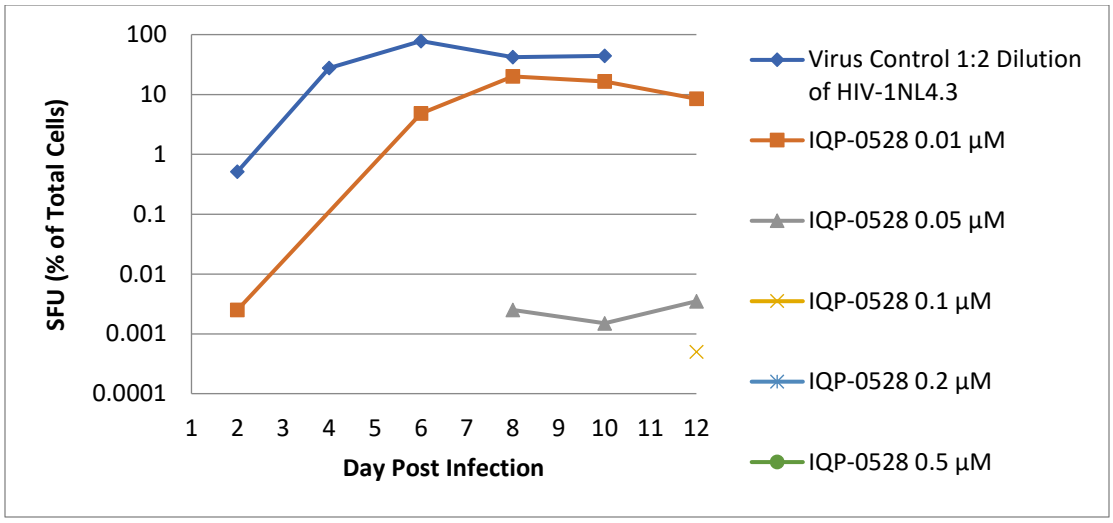


Figure 14A. Percentage of total cells infected with HIV-1_{NL4-3} in the ICCA at varying concentrations of IQP-0528 - Experiment one. The 0.01 µM treated culture had peak infection on day 8. Cultures with 0.05 µM or greater IQP-0528 concentrations were at or below the limit of detection defined in the SFU assay.

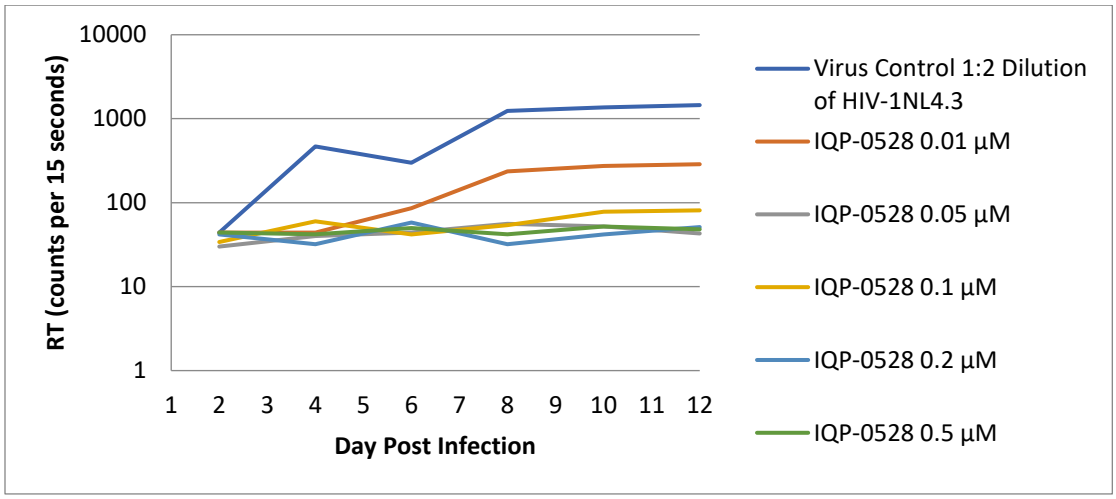


Figure 14B. RT observed in cell cultures infected with HIV-1_{NL4-3} in the presence of IQP-0528 at varying concentrations - Experiment one. The 0.01 µM treated culture showed increased RT compared to the other cultures from day 6 to day 12 of the ICCA. All other cultures carried in the presence of IQP-0528 had RT comparable to uninfected CEM-SS cells.

In a second confirmatory experiment, the IQP-0528 concentrations evaluated included 0.01 μM, 0.05 μM, 0.1 μM and 0.2 μM IQP-0528. These concentrations were selected based on the results from the first experiment, and the highest concentration evaluated was 0.2 μM because this culture remained uninfected.

The 0.01 μM treated culture had peak infection on day 8 with greater than 10% of the cells in the culture infected. The 0.05 μM, 0.1 μM and 0.5 μM IQP-0528 treated cultures remained negative for virus infection through the 12 days of the ICCA. These data are presented in Figure 15A.

RT in cell free supernatants for all cultures including the virus control were low. Peak RT was observed on day 12 for the virus control culture. Cultures carried in the presence of IQP-0528 at all concentrations had RT levels comparable to uninfected CEM-SS cells. These data are presented in Figure 15B.

These results suggest that the sterilizing concentration of IQP-0528 in cultures exposed to HIV-1_{NL4-3} is 0.05 μM or greater.

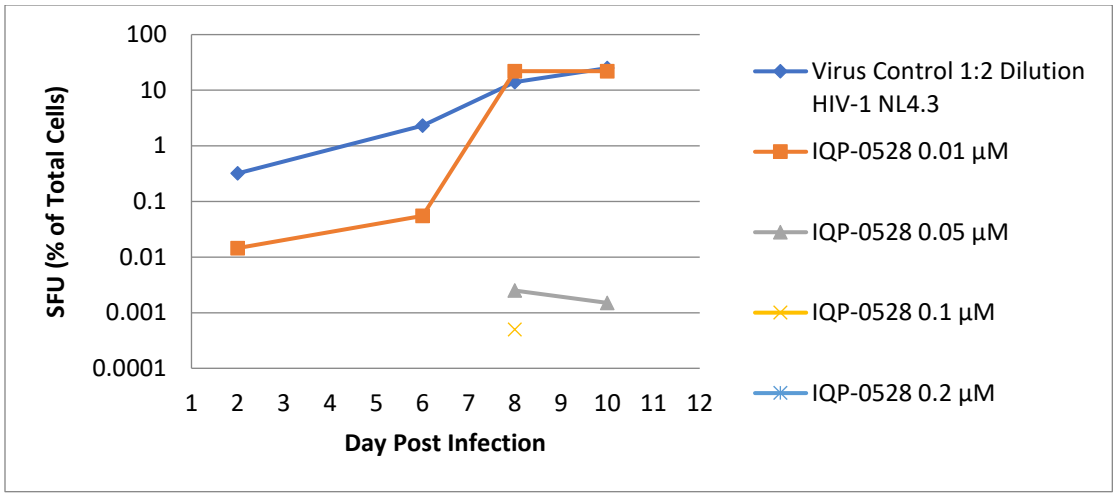


Figure 15A. Percentage of total cells infected with HIV-1_{NL4-3} in the ICCA at varying concentrations of IQP-0528 - Experiment Two. The 0.01 µM treated culture had peak infection on day 8 with greater than 10% of the cells in the culture infected. All other cultures carried in the presence of IQP-0528 had infection that was below the limit of detection.

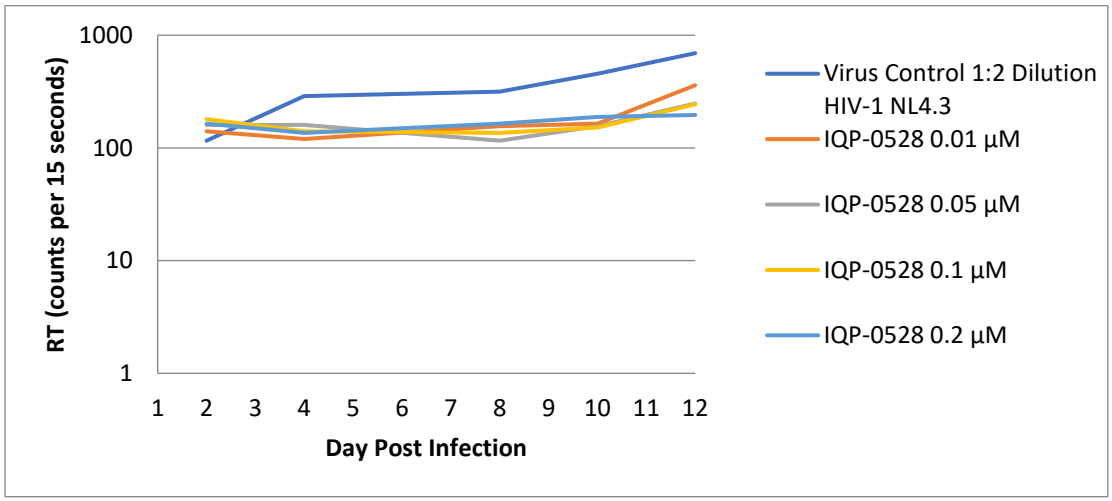


Figure 15B. RT in cell cultures infected with HIV-1_{NL4-3} in the presence of IQP-0528 at varying concentrations - Experiment two. Peak RT was observed at day 12 for the virus control. Cultures treated with all concentrations of IQP-0528 had RT equivalent to background levels.

Determination of the Effect of MOI on the Sterilizing Concentration of IQP-0528 in the ICCA

To determine the effect of varying MOI on the sterilizing concentration of IQP-0528, two ICCAs were performed at three different MOIs with three concentrations of IQP-0528. In both ICCAs, the ICCA method was used with the following variations: HIV-1_{IIIB} dilutions of 1:1 (neat), 1:5 and 1:50 were used with IQP-0528 concentrations of 0.05 μM, 0.1 μM and 0.2 μM. These cultures were monitored for cell density and viability (not shown), syncytium formation and measurement of RT in cell free supernatant.

Virus Control Cultures

The cultures infected with a 1:1 and 1:5 dilution of virus had peak infection on day 4 of the assay with 100% of the cultures infected. The cultures infected with a 1:50 dilution of virus had peak infection on day 6 with 100% of the culture infected. These data are shown in Figures 16A, C and E. Cultures infected with 1:1 and 1:5 dilutions of virus had peak RT on day 4 of the assay. The culture infected with a 1:50 dilution of virus had peak RT on day 6. These data are shown in Figure 16B, D and F.

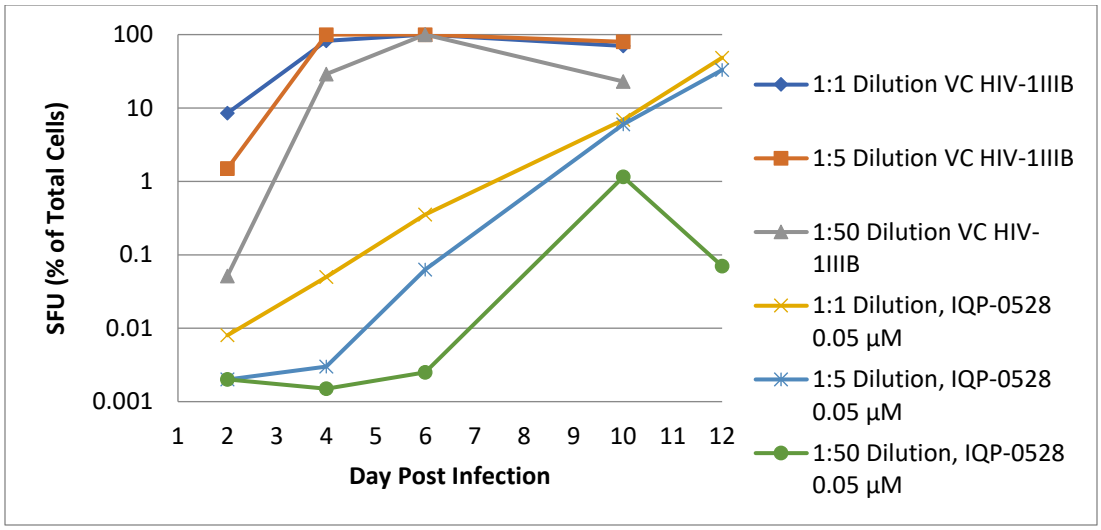
Cultures in the Presence of IQP-0528

All cultures grown in the presence of 0.05 μM IQP-0528 became infected before day 12 of the assay. The cultures infected with a 1:1 and 1:5 dilution of virus had peak infection on day 12 with greater than 50% of the cultures infected. The culture infected with a 1:50 dilution of virus had peak infection on day 10 of the assay with 1% of the cells in the culture infected. These data are shown in Figure 16A. Peak RT in the cultures infected with a 1:1 and 1:5 dilution of virus was observed on day 12. RT in the culture infected with a 1:50 dilution of virus did not reach above background levels. These data

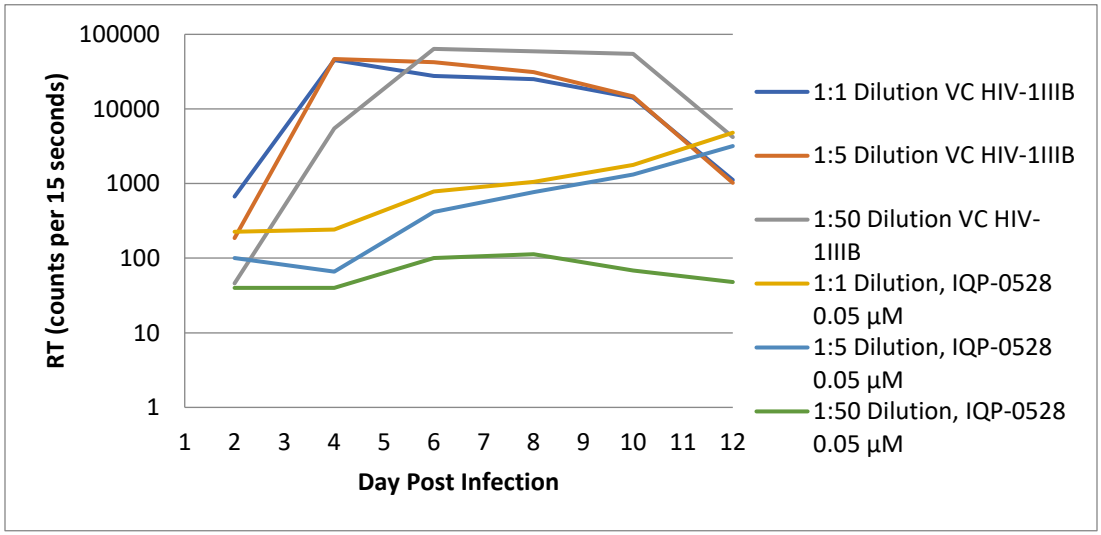
are shown in Figure 16B. In order to more sensitively evaluate infection of the target cells and to confirm culture sterilization, we also adapted a PCR based quantitative assay to look at HIV in infected cells. All of these cultures were also confirmed to be infected by PCR. These data are shown in Figure 16G.

The cultures infected with a 1:1 and 1:5 dilution of virus and grown in the presence of 0.1 μM IQP-0528 had peak infection on day 12, with less than 1% of the cells in the culture infected. The culture infected with a 1:50 dilution of virus remained below the limit of detection in the SFU assay. These data are shown in Figure 16C. All cultures had RT at background levels for the duration of the ICCA. These data are shown in Figure 16D. PCR performed on samples from the cultures confirmed that the cultures infected with a 1:1 and 1:5 dilution of virus were infected on day 12 of the ICCA and the cultures infected with a 1:50 dilution of virus remained uninfected. Post compound removal, the culture infected with a 1:50 dilution remained uninfected which was confirmed with visual observation of CPE in the cultures and PCR.

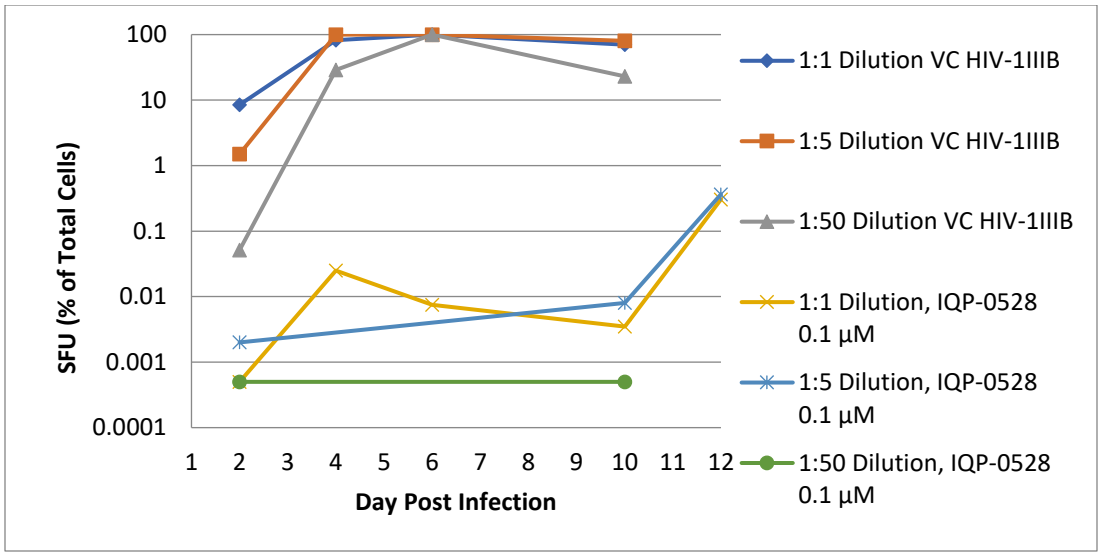
One syncytium count was observed to be above the lower limit of detection in the 1:5 dilution of virus grown in the presence of 0.2 μM on day 12. All other cultures grown in the presence of 0.2 μM IQP-0528 were below the limit of detection for infected cells in the SFU assay. These data are shown in Figure 16E. RT was at background levels for the duration of the ICCA for these cultures (Figure 16F) and all cultures were confirmed to be uninfected by PCR. (Figure 16G). Post compound removal, all cultures remained negative for virus infection and the results were confirmed by visual observation of CPE in the cultures and PCR.



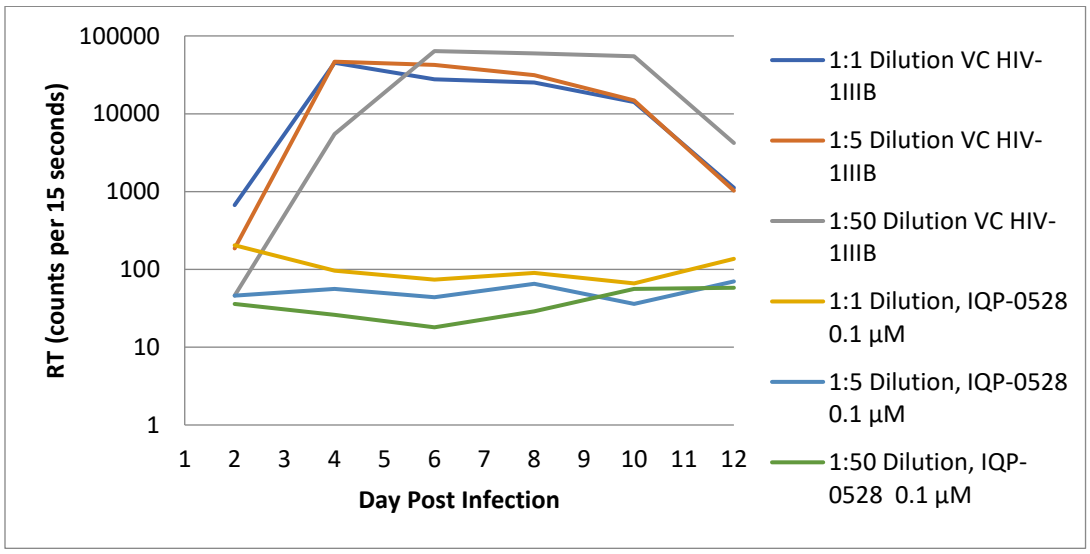
Figures 16A. Percentage of infected cells in HIV-1_{IIIB} cultures grown in the presence of 0.05 µM IQP-0528 at varying MOIs – Experiment one. The 0.05 µM treated cultures infected with a 1:1 and 1:5 dilution of virus had peak infection on day 12 of the assay. The 0.05 µM treated culture infected with a 1:50 dilution of virus had peak infection on day 10.



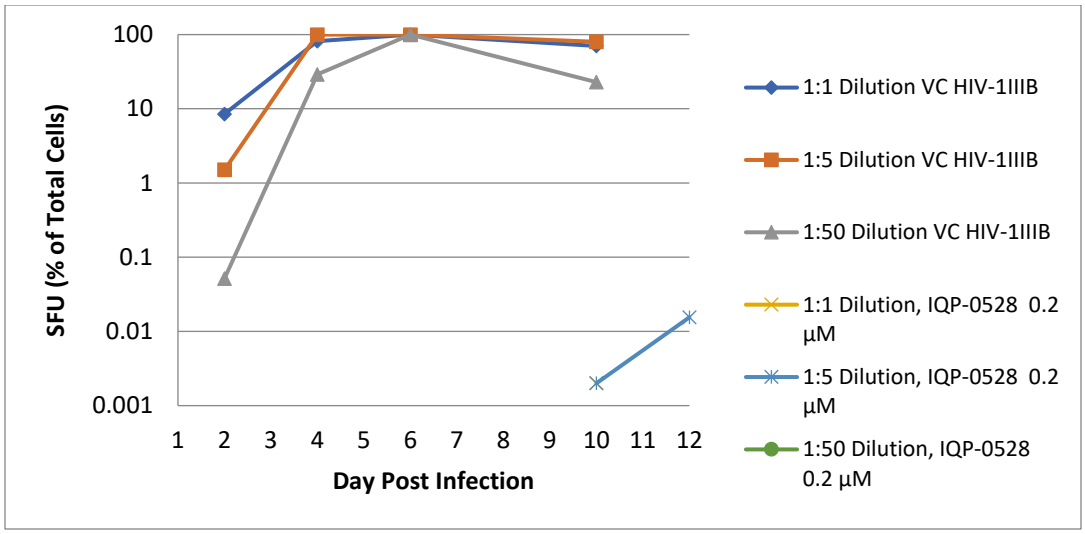
Figures 16B. RT in HIV-1_{IIIB} cultures grown in the presence of 0.05 µM IQP-0528 at varying MOIs – Experiment one. The 0.05 µM treated cultures infected with a 1:1 and 1:5 dilution of virus had peak RT on day 12. The culture diluted with a 1:50 dilution of virus had no RT above background levels.



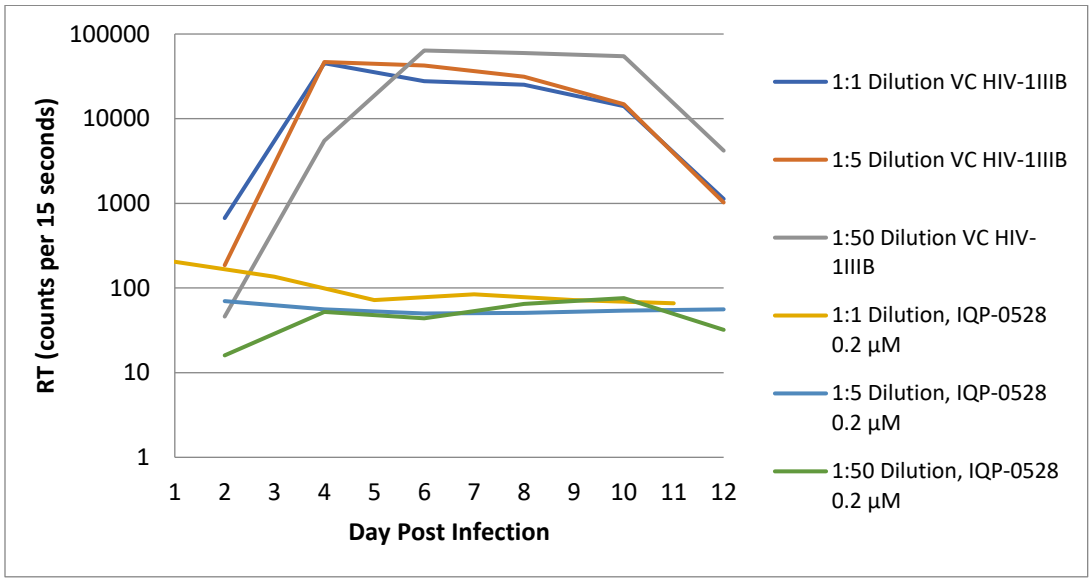
Figures 16C. Percentage of infected cells in HIV-1_{IIIB} cultures grown in the presence of 0.1 µM IQP-0528 at varying MOIs – Experiment one. The 0.1 µM treated cultures infected with a 1:1 and 1:5 dilution of virus had peak infection on day 12. The 0.1 µM treated culture infected with a 1:50 dilution of virus remained below the detectable limit for the ICCA.



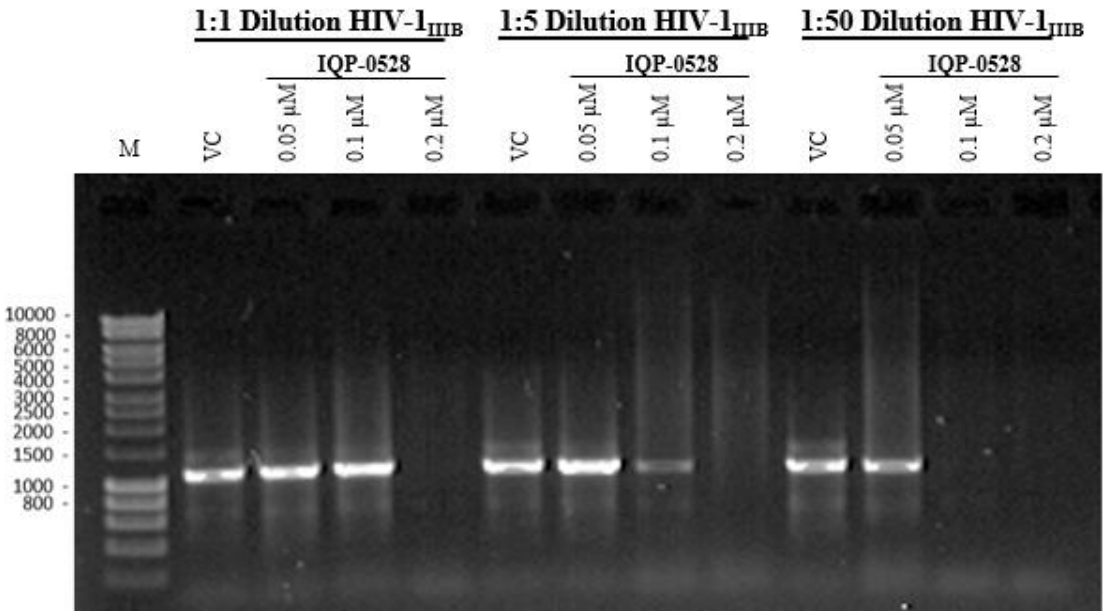
Figures 16D. RT in HIV-1_{IIIB} cultures grown in the presence of 0.1 µM IQP-0528 at varying MOIs – Experiment one. RT for all cultures remained below background levels.



Figures 16E. Percentage of infected cells in HIV-1_{IIIB} cultures grown in the presence of 0.2 µM IQP-0528 at varying MOIs – Experiment one. One syncytium count above the lower limit of detection was observed in 0.2 µM treated culture infected with a 1:5 dilution of virus on day 12. All other cultures remained below the detectable limit for infected cells.



Figures 16F. RT in HIV-1_{IIIB} cultures grown in the presence of 0.2 µM IQP-0528 at varying MOIs – Experiment one. RT for all cultures remained at background levels.



Figures 16G. PCR Results of the HIV-1_{IIIB} cultures grown in the presence of varying concentrations of IQP-0528 at varying MOIs – Experiment one. Lane 2: Positive PCR result of the 1:1 virus control culture on day 12 of the ICCA. Lanes 3 – 4: Positive PCR result for the 0.05 µM and 0.1 µM IQP-0528 treated cultures at a 1:1 dilution of virus at day 12 of the ICCA. Lane 5: Negative PCR result for the 0.2 µM IQP-0528 treated culture at a 1:1 virus dilution at day 12 of the ICCA. Lane 6: Positive PCR result for the 1:5 virus control culture on day 12 of the ICCA. Lanes 7- 8: Positive PCR result for the 0.05 µM and 0.1 µM IQP-0528 treated cultures at a 1:5 dilution of virus at day 12 of the ICCA. Lane 9: Negative PCR result of the 0.2 µM IQP-0528 treated culture at a 1:5 dilution of virus on day 12 of the ICCA. Lane 10: Positive PCR result of the 1:50 virus control culture on day 12 of the ICCA. Lanes 11: Positive PCR result for the 0.05 µM treated culture at a 1:50 dilution of virus at day 12 of the ICCA. Lanes 12 - 13: Negative PCR result for the 0.1 µM and 0.2 µM IQP-0528 treated cultures at a 1:50 dilution of virus at day 12 of the ICCA

Experiment #2

Virus Control Cultures

In the second assay, the cultures infected with a 1:1 dilution of virus had peak infection on day 4 with 100% of the culture infected. The cultures infected with a 1:5 and 1:50 dilution of virus had peak infection on day 6 with 100% of the culture infected. These data are shown in Figure 17A, C and E. The culture infected with a 1:1 dilution of virus had peak RT on day 4. The cultures infected with a 1:5 and 1:50 dilution of virus had peak RT on day 6. These data are shown in Figure 17B, D, and F.

Cultures in the Presence of IQP-0528

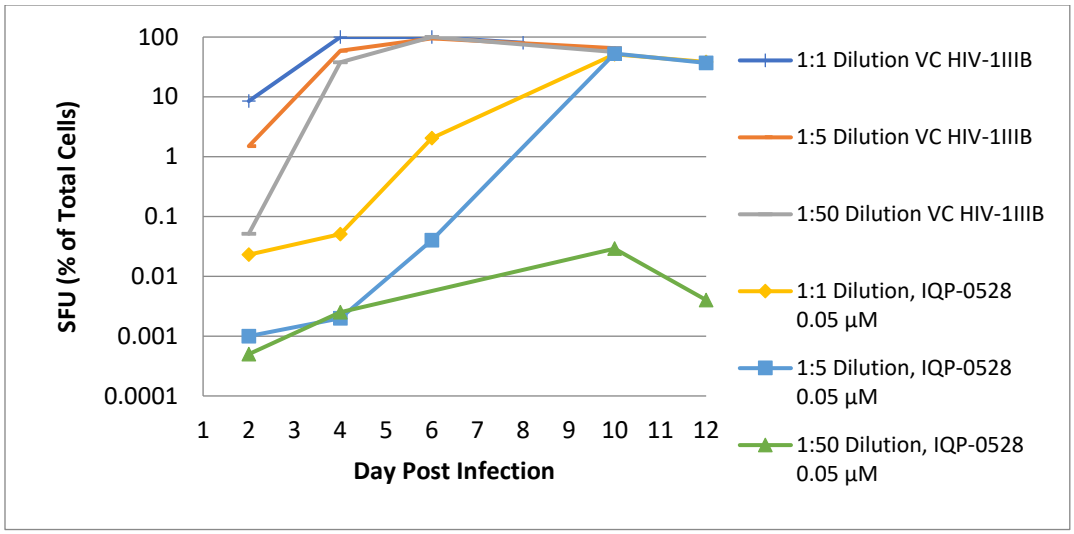
All cultures grown in the presence of 0.05 µM IQP-0528 became infected before day 12 of the assay. The cultures infected with a 1:1 and 1:5 dilution of virus had peak infection on day 10 with greater than 50% of the cells in the cultures infected. The culture infected with a 1:50 dilution of virus had peak infection on day 10 of the assay with 0.1% of the culture infected. These data are shown in Figure 17A. Peak RT for the cultures infected with a 1:1 dilution of virus was observed on day 10. Peak RT in the culture infected with a 1:5 dilution of virus was observed on day 12. The culture infected with a 1:50 dilution of virus had RT below background levels. These data are shown in Figure 17B. All cultures were confirmed positive for infection by PCR. These results are shown in Figure 17G.

The cultures infected with a 1:1 dilution of virus and grown in the presence of 0.1 µM IQP-0528 had peak infection on day 12, with 10% of the cells infected. The cultures infected with a 1:5 and 1:50 dilution of virus remained below the limit of detection in the SFU assay. These data are presented in Figure 17C. Peak RT was observed on day 12

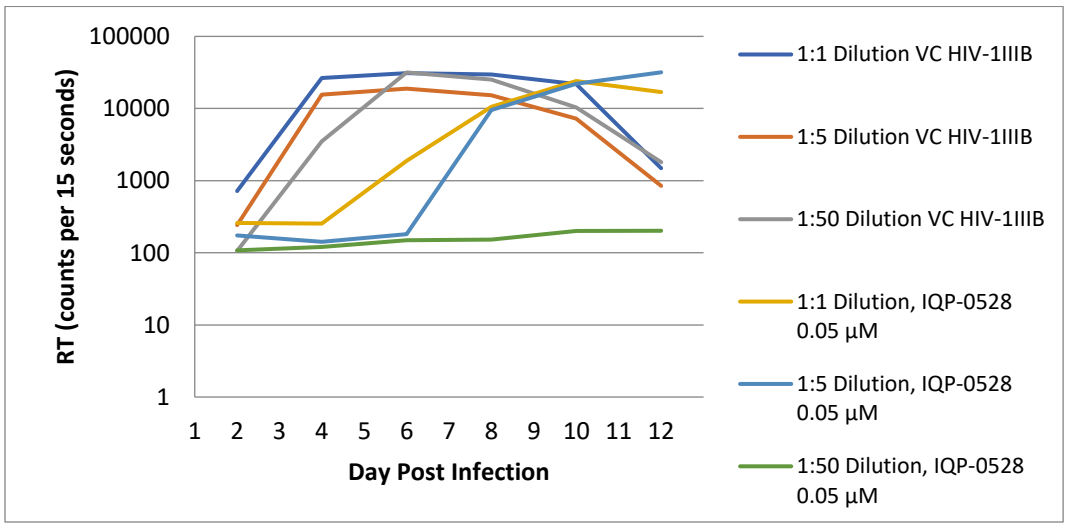
in the cultures infected with a 1:1 dilution of virus. The cultures infected with a 1:5 and 1:50 dilution of virus had RT at background levels for the duration of the ICCA. These data are presented in Figure 17D. Confirmatory PCR performed on the samples concluded that the cultures infected with a 1:1 and 1:5 dilution of virus were infected, and the culture infected with a 1:50 dilution of virus remained uninfected. Post compound removal, the culture infected with a 1:5 dilution of virus was positive for CPE in the cultures on day 5 post compound removal (day 17 of the ICCA). The culture infected with a 1:50 dilution of virus remained negative for virus infection which was confirmed by lack of CPE in the cultures and PCR.

All cultures grown in the presence of 0.2 µM IQP-0528 were below the limit of detection for infected cells in the SFU assay. These data are shown in Figure 17E. All cultures had background levels of RT for the duration of the ICCA. These data are shown in Figure 17F. All cultures were confirmed to be uninfected by PCR. These results are presented in Figure 17G. Post compound removal, the cultures infected with a 1:5 dilution of virus became infected which was confirmed through visualization of CPE in the cultures on day 5 post compound removal (day 17 of the ICCA). All other cultures remained negative for infection as measured by CPE in the cultures and PCR through 30 days after compound removal.

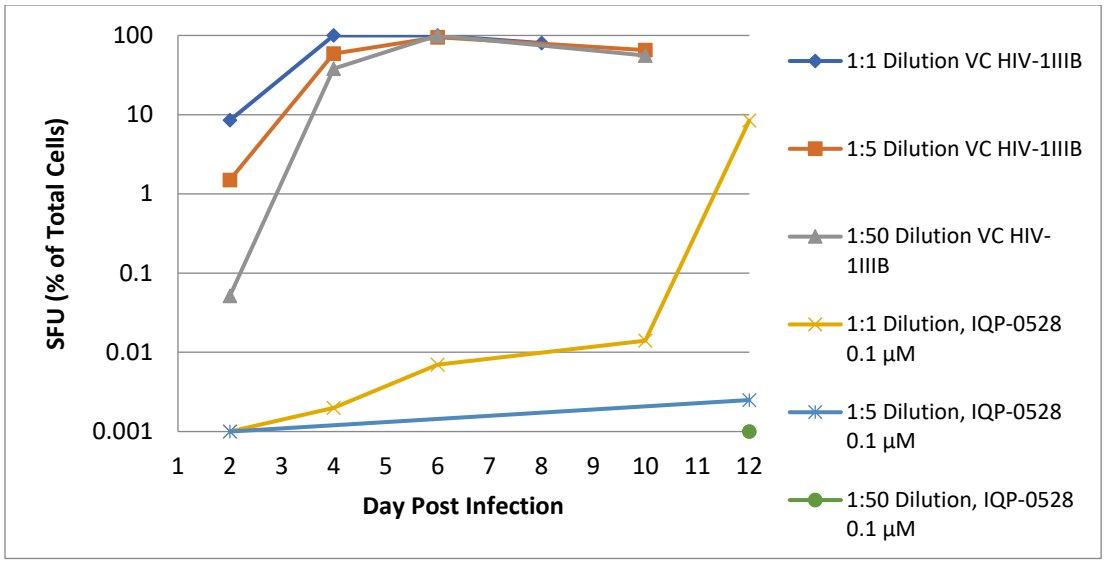
These results suggest that the activity of IQP-0528 is MOI dependent and that the IQP-0528 concentration needs to be increased when challenged with higher MOIs of virus in order to sterilize the culture.



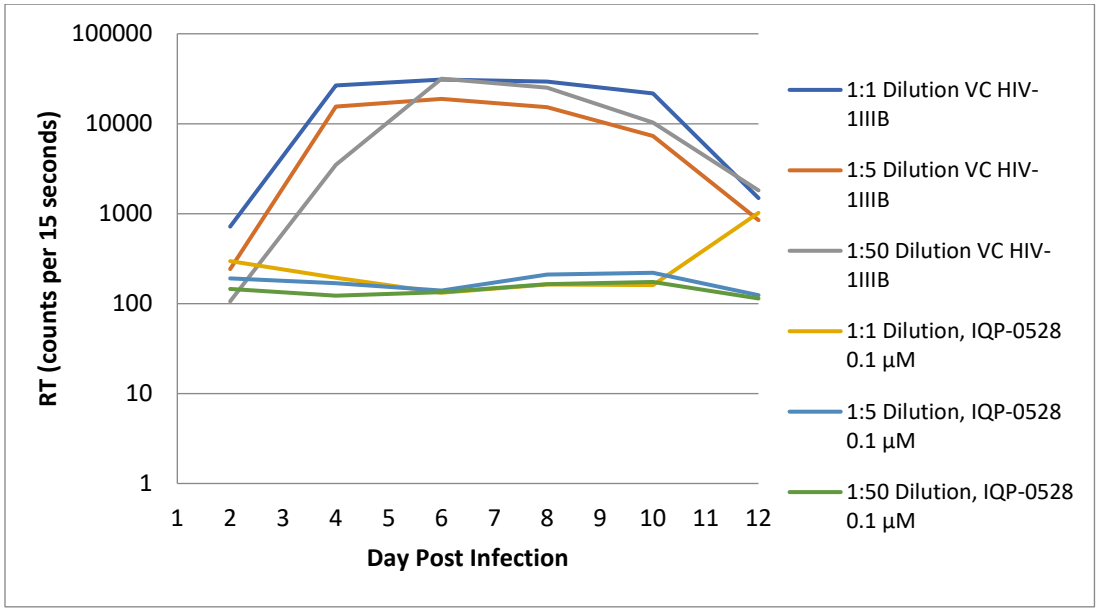
Figures 17A. Percentage of infected cells in HIV-1_{IIIB} cultures grown in the presence of 0.05 µM IQP-0528 at varying MOIs– Experiment two. The cultures infected with a 1:1 and 1:5 dilution of virus peaked in infection on day 10. The culture infected with a 1:50 dilution of virus had peak infection on day 10.



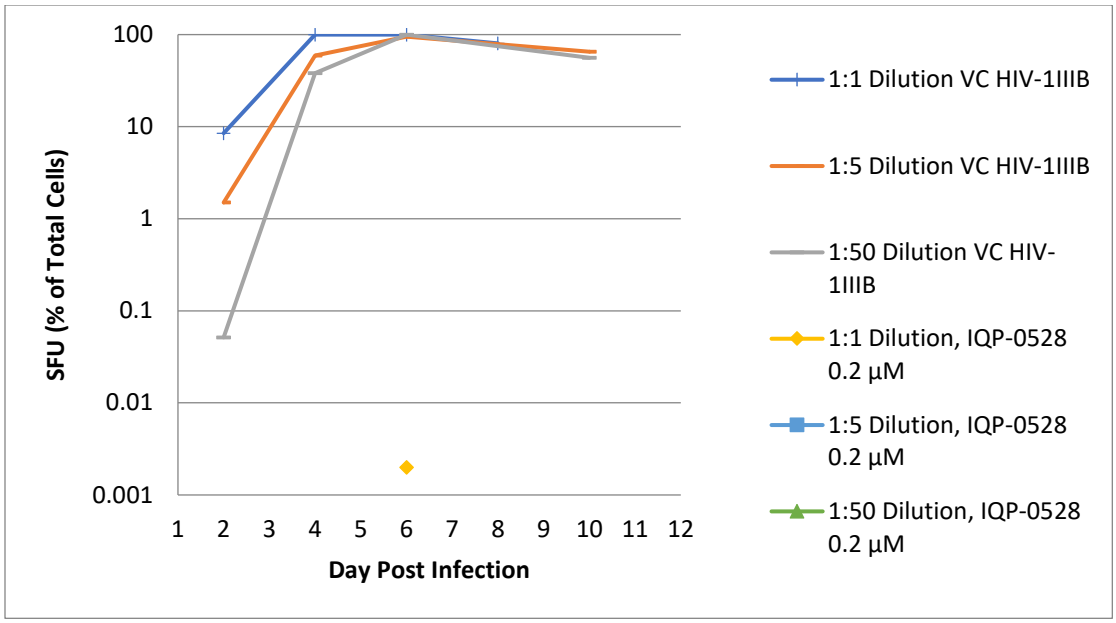
Figures 17B. RT in HIV-1_{IIIB} cultures grown in the presence of 0.05 µM IQP-0528 at varying MOIs – Experiment two. The culture infected with a 1:1 dilution of virus had peak RT on day 10. The culture infected with a 1:5 dilution of virus had peak RT on day 12. The culture infected with a 1:50 dilution of virus had RT equivalent to background levels.



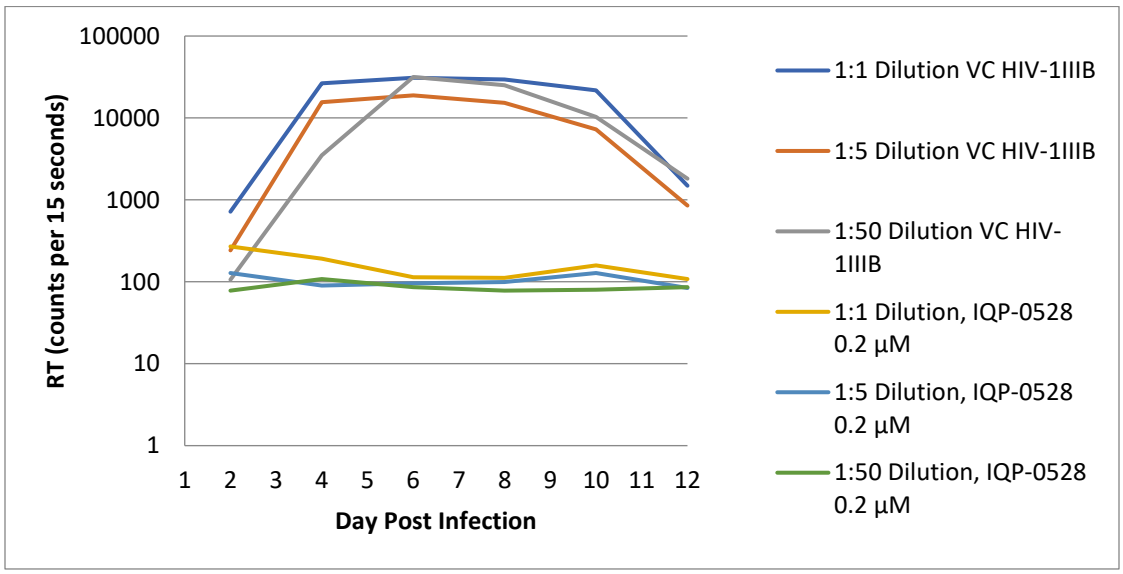
Figures 17C. Percentage of infected cells in HIV-1_{IIIB} cultures grown in the presence of 0.1 µM IQP-0528 at varying MOIs – Experiment two. The culture infected with a 1:1 dilution of virus had peak infection on day 12. The culture diluted with a 1:5 and 1:50 dilution of virus remained below the detectable limit through the duration of the ICCA.



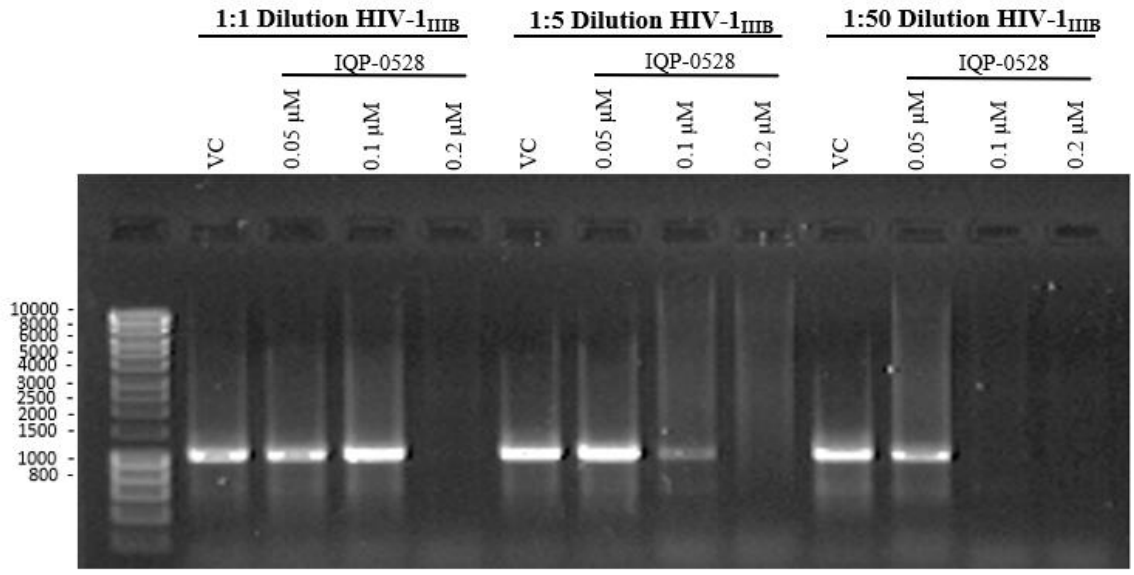
Figures 17D. RT in HIV-1_{IIIB} cultures grown in the presence of 0.1 µM IQP-0528 at varying MOIs – Experiment two. The cultures infected with a 1:1 dilution of virus had peak RT on day 12. The cultures infected with a 1:5 and 1:50 dilution of virus had RT equivalent to background levels.



Figures 17E. Percentage of infected cells in HIV-1_{IIIB} cultures grown in the presence of 0.2 µM IQP-0528 at varying MOIs – Experiment two. All cultures remained below the detectable limit for infected cells through the duration of the ICCA.



Figures 17F. RT in HIV-1_{IIIB} cultures grown in the presence of 0.2 µM IQP-0528 at varying MOIs – Experiment two. All cultures had RT equivalent to background levels through the duration of the ICCA.



Figures 17G. PCR Results of the HIV-1_{IIIB} cultures grown in the presence of varying concentrations of IQP-0528 at varying MOIs – Experiment two. Lane 2: Positive PCR result for the 1:1 MOI virus control culture on day 12 of the ICCA. Lanes 3 – 4: Positive PCR result for the 0.05 µM and 0.1 µM IQP-0528 treated cultures at a 1:1 dilution of virus at day 12 of the ICCA. Lane 5: Negative PCR result for the 0.2 µM IQP-0528 treated culture at a 1:1 virus dilution at day 12 of the ICCA. Lane 6: Positive PCR result for the 1:5 virus control culture on day 12 of the ICCA. Lanes 7- 8: Positive PCR result for the 0.05 µM and 0.1 µM IQP-0528 treated cultures at a 1:5 dilution of virus at day 12 of the ICCA. Lane 9: Negative PCR result of the 1:5 MOI virus control culture on day 12 of the ICCA. Lane 10: Positive PCR result of the 1:50 virus control culture on day 12 of the ICCA. Lane 11: Positive PCR result for the 0.05 µM treated culture at a 1:50 dilution of virus at day 12 of the ICCA. Lanes 12- 13: Negative PCR result for the 0.1 µM and 0.2 µM IQP-0528 treated cultures at a 1:50 dilution of virus at day 12 of the ICCA

Determination of the Effect of Pretreatment of IQP-0528 on HIV-1_{IIIB} Transmission in the ICCA

To determine the effect of pretreatment with IQP-0528 on cultures infected with HIV-1_{IIIB} in the ICCA and to determine the minimum pretreatment time needed to prevent infection, duplicate ICCAs were performed. The first ICCA was performed according to the ICCA method with the following variations: CEM-SS cells were incubated with 0.05 μM, 0.1 μM or 0.2 μM IQP-0528 for 1, 2, 4 or 8 hours prior to infection with HIV-1_{IIIB}. HIV-1_{IIIB} was used at a virus dilution of 1:50 to infect the pretreated CEM-SS cells. IQP-0528 was diluted in the cultures instead of removed to simulate the natural progression of compound through the female genital tract, where compound would be gradually diluted according to its tissue half-life. These cultures were monitored for cell density and viability (not shown), syncytium formation and measurement of RT in the cell free supernatants.

Pretreatment with 0.05 μM and 0.1 μM IQP-0528 had no impact on the transmission of HIV-1_{IIIB}. All cultures had peak infection on day 5 of the ICCA with greater than 50% of the cultures infected. RT peaked on day 5 for all cultures. These data are presented in Figure 18A, B, C and D.

Pretreatment with 0.2 μM IQP-0528 also had no impact on the transmission of HIV-$1_{IIIB.}$ All cultures had peak infection on day 5, with greater than 10% of the cultures infected. RT peaked for all cultures on day 5 of the assay. These data are presented in Figures 18E and F.

These results suggest that pretreatment with IQP-0528 for up to 8 hours has no effect on virus transmission in cultures where the sterilizing concentration of IQP-0528 is

not maintained after infection. The residual concentration of IQP-0528 in the cultures is not sufficient to prevent transmission and cause sterilization.

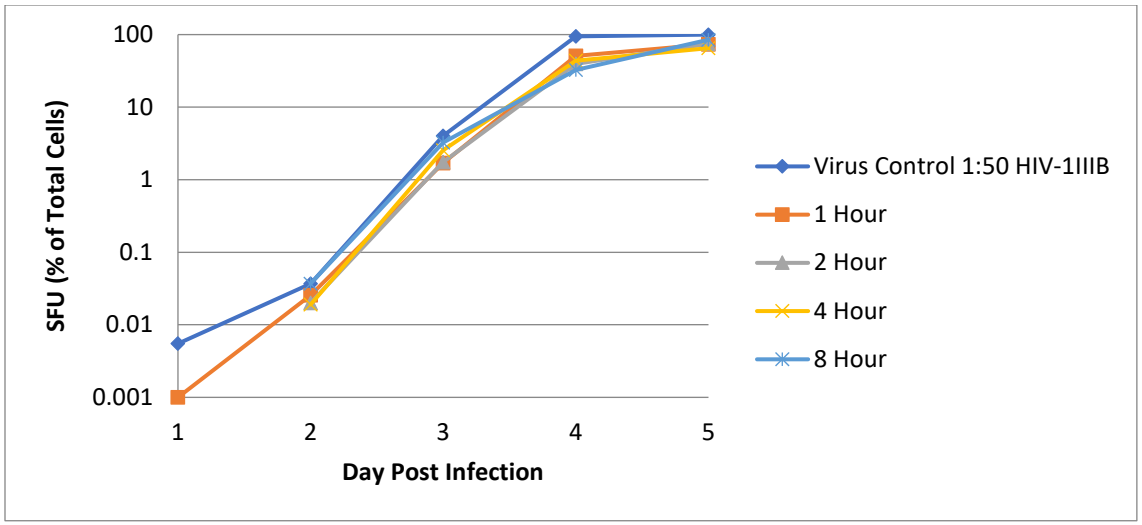


Figure 18A. Percentage of infected cells in the ICCA cultures pretreated with 0.05 µM IQP-0528 at various timed intervals - Experiment one. Pretreatment with 0.05 µM for 8-hours or less with no additional IQP-0528 had no effect on virus transmission. Peak day of infection for all cultures was day 5.

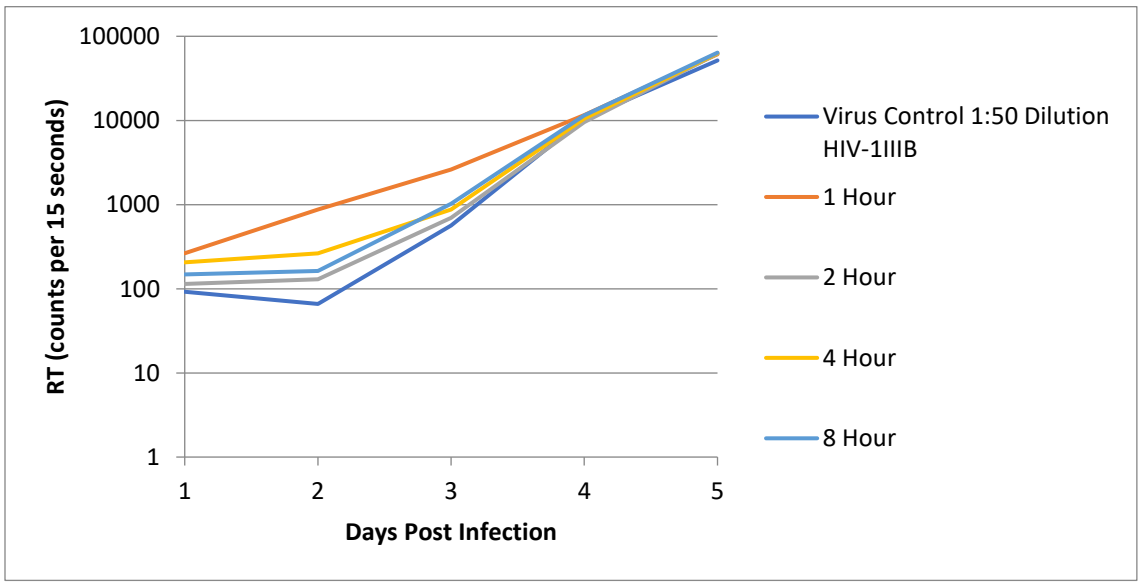


Figure 18B. RT in ICCA cultures pretreated with 0.05 µM IQP-0528 at various time intervals – Experiment one. The culture pretreated with 0.05 µM for 8 hours or less with no additional IQP-0528 added had peak RT on day 5.

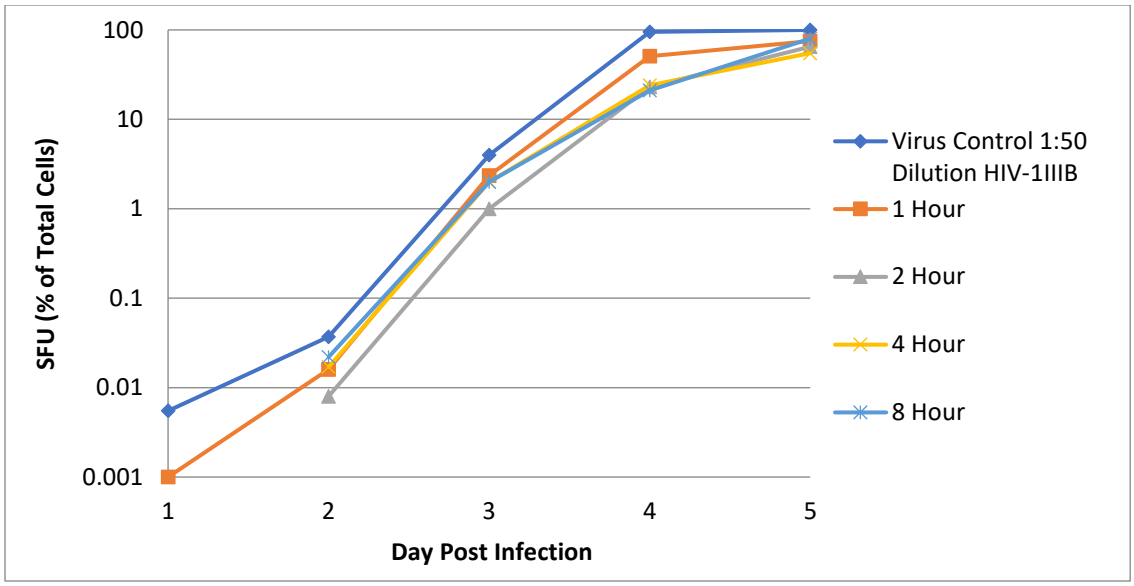


Figure 18C. Percentage of infected cells in the ICCA cultures pretreated with 0.1 µM IQP-0528 at various time intervals – Experiment one. Peak infection was observed on day 5 for all cultures.

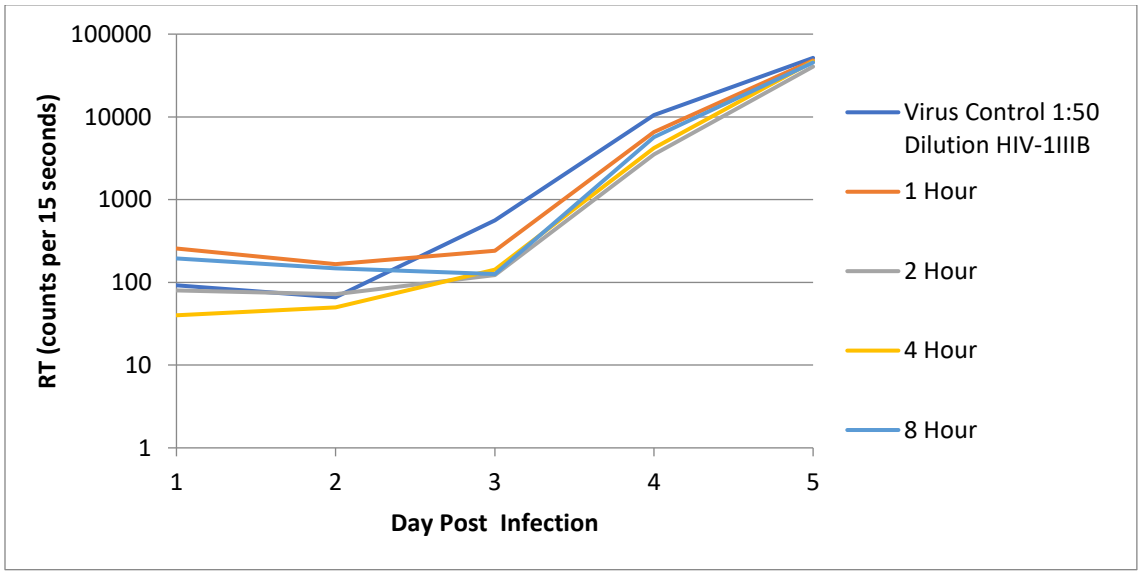


Figure 18D. RT in the ICCA cultures pretreated with 0.1 µM IQP-0528 at various time intervals – Experiment one. Peak RT was observed on day 5 for all cultures.

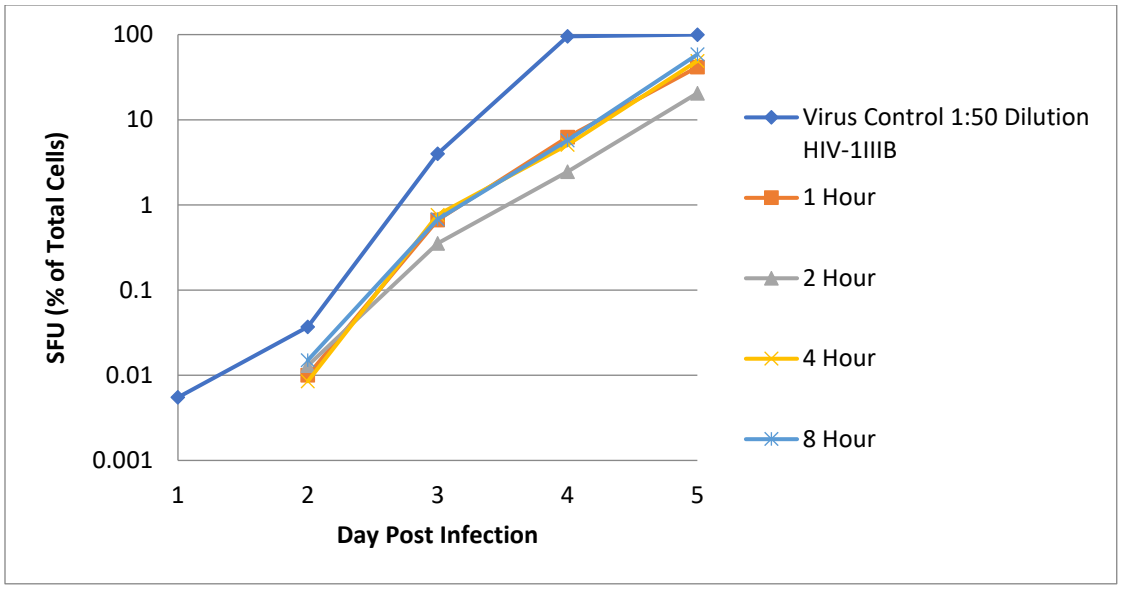


Figure 18E. Percentage of infected cells in the ICCA cultures pretreated with 0.2 µM IQP-0528 at various time intervals – Experiment one. Peak infection for all cultures was seen on day 5.

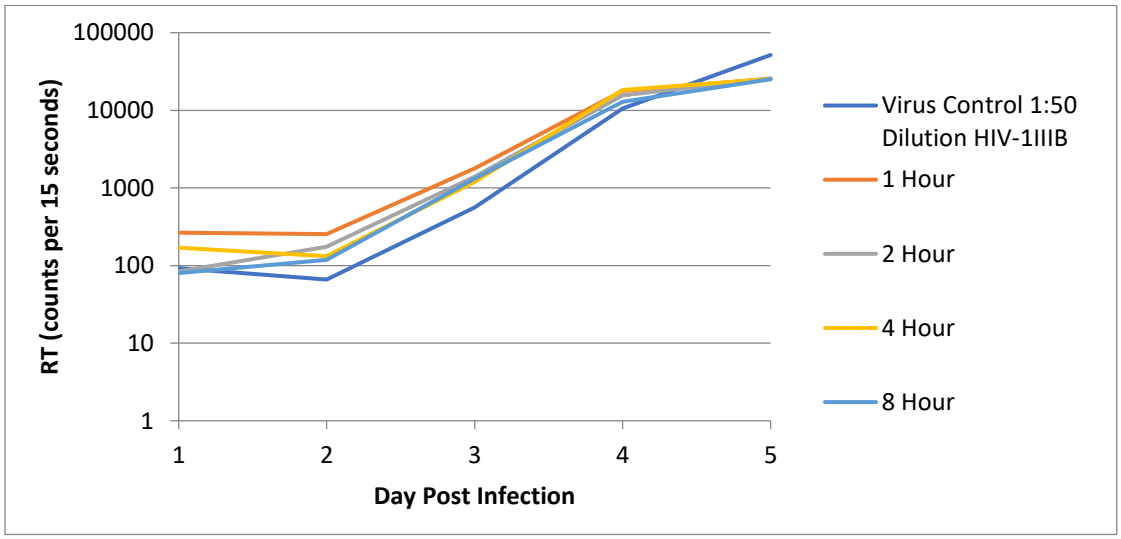


Figure 18F. RT in the ICCA cultures pretreated with 0.2 µM IQP-0528 at various time intervals – Experiment one. Peak RT for all cultures was observed on day 5.

The second ICCA used to determine the effect of pretreatment on the transmission of HIV-1_{IIIB} was performed according to the ICCA method with the following variations: CEM-SS cells were incubated with 1 μM, 5 μM, 10 μM, 15 μM or 20 μM IQP-0528 for 2 hours prior to infection with HIV-1_{IIIB}. HIV-1_{IIIB} was used at a virus dilution of 1:50. Following a twenty-four hour incubation, the volume of the cultures was adjusted such that the final concentration of IQP-0528 in the cultures was 0.02 μM, 0.1 μM, 0.2 μM, 0.3 μM and 0.4 μM for 1 μM, 5 μM, 10 μM, 15 μM or 20 μM IQP-0528, respectively. These cultures were monitored for cell density and viability (not shown), syncytium formation and RT in the cell free supernatant.

Pretreatment of the cultures with an initial concentration of 1 μM IQP-0528 followed by dilution to 0.02 μM had little effect on virus transmission in the cultures, with peak infection occurring on day 8 with 90% of the cells in the culture infected. Pretreatment with initial concentrations of 5 μM or greater followed by culturing in the presence of concentrations of IQP-0528 greater than 0.1 μM resulted in suppression in virus transmission which was confirmed with SFU counts below the limit of detection. These data are presented in Figure 19A.

Peak RT was observed on day 8 in the 1 μM culture. All other cultures had RT at background levels. These data are presented in Figure 19B.

On day 12 of the assay, the cultures remaining negative for CPE were washed to remove residual IQP-0528. All cultures remained negative as observed by visual CPE of the cultures through 40 days of the ICCA.

These results suggest that pretreatment with IQP-0528 at 5 μM or greater for 2 hours is adequate to suppress virus transmission with a residual IQP-0528 concentration of 0.1 μM or greater, which coincides with the sterilizing concentration.

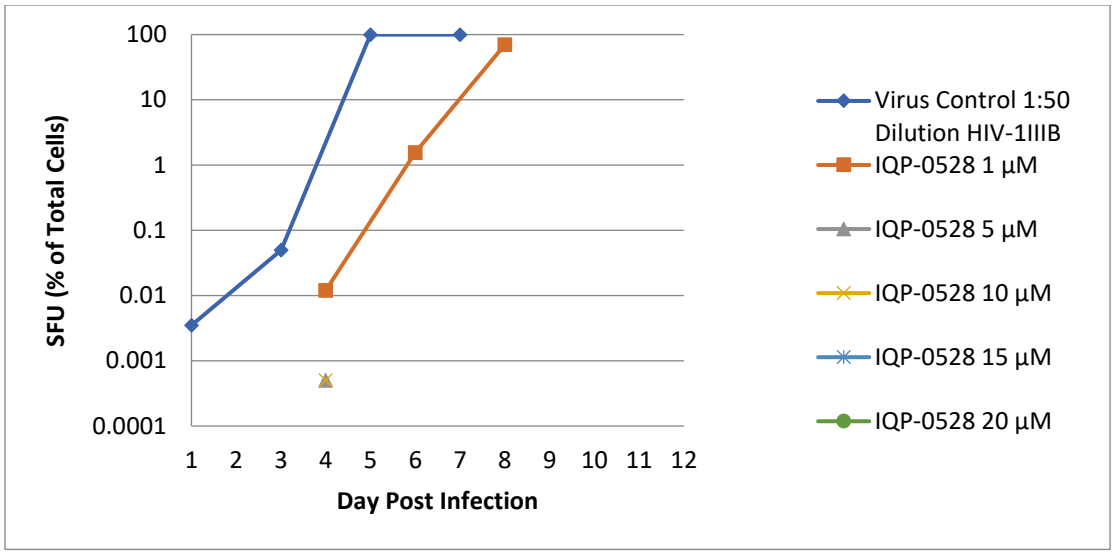


Figure 19A. Percentage of infected cells in the ICCA cultures pretreated with varying IQP-0528 concentrations for two hours prior to infection - Experiment two. The culture pretreated with 1 µM IQP-0528 showed peak infection on day 8. All other cultures remained below the detectable limit of the assay.

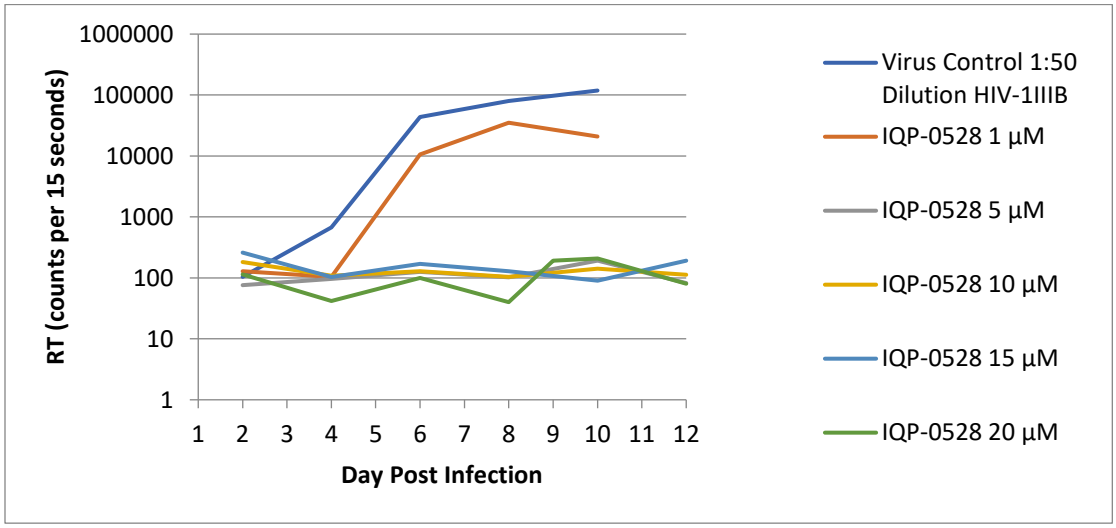


Figure 19B. RT in the ICCA cultures pretreated with varying IQP-0528 concentrations for two hours prior to infection - Experiment two. The culture pretreated with 1 µM IQP-0528 had peak RT at day 8 of the assay. All other cultures remained at background levels of RT.

To determine the effect of pretreating the ICCA cultures with specified concentrations of IQP-0528 followed by removal of IQP-0528, a third ICCA was performed. The ICCA method was followed with the following variations: CEM-SS cells were pretreated with IQP-0528 at 1 μM, 5 μM, 10 μM, 15 μM and 20 μM for 2 hours prior to infection with a 1:50 dilution of HIV-1_{IIIB}. Following the incubation, the cultures were diluted such that the final concentration of IQP-0528 was 0.04 μM, 0.2 μM, 0.4 μM, 0.8 μM and 1.6 μM, respectively. Following an additional 1-hour incubation, the cultures were washed three times to remove residual IQP-0528 and the cells were resuspended in assay media in the absence of any IQP-0528. These cultures were monitored for cell density and viability (not shown), syncytium formation and RT in the cell free supernatant.

All cultures had peak infection on day 8 of the assay with greater than 90% of the cells in all the cultures infected. These data are shown in Figure 20A. The 10 μM and 15 μM treated cultures had peak RT on day 8. All other cultures had peak RT on day 10 of the assay. These data are shown in Figure 20B.

These results suggest that pretreatment with IQP-0528 does not work when the compound is removed at 1 hour post infection, even at high concentrations. Even when pretreating cultures with concentrations of IQP-0528 well above the sterilizing concentration, transmission still occurs after compound has been removed from the cultures. These data support that IQP-0528 must remain in culture following a pretreatment at concentrations at or greater than the sterilizing concentration.

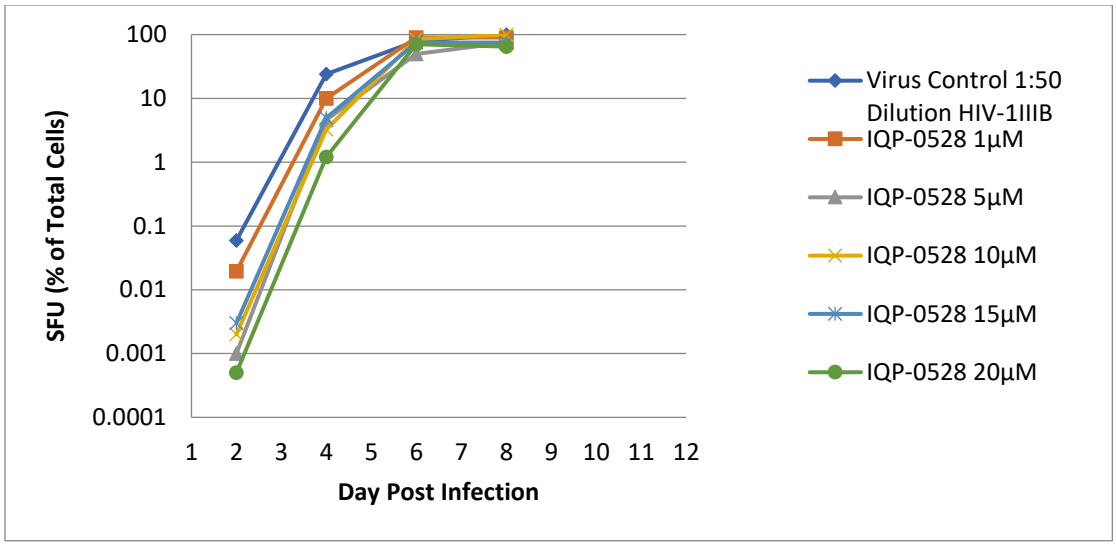


Figure 20A. Percent of infected cells in the ICCA cultures pretreated with varying IQP-0528 concentrations with removal of compound at 1-hour post infection. Post compound removal, all cultures had peak infection on day 8.

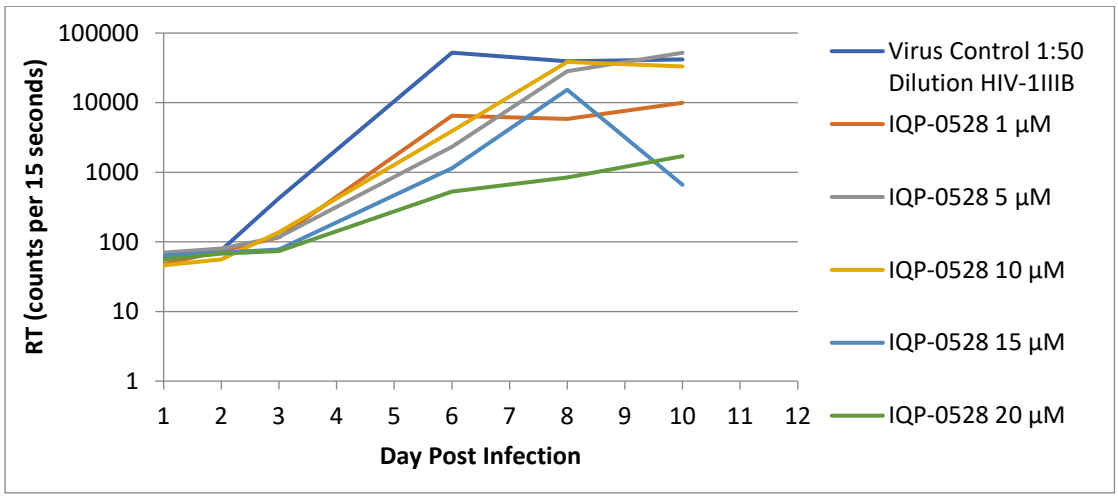


Figure 20B. RT in the ICCA cultures pretreated with varying IQP-0528 concentrations with removal of compound at 1-hour post infection. Peak RT for all cultures was seen on day 10, except the 10 µM and 15 µM treated cultures which showed peak activity on day 8.

Determining the Effect of Delayed Addition of IQP-0528 Following Infection With HIV-1_{IIIB}

To determine the effect of the delayed addition of IQP-0528 on the viral replication kinetics of HIV-1_{IIIB}, two ICCAs were performed. In these experiments the addition of IQP-0528 was delayed by 24, 48, 72 and 96 hours post infection. Based on the data from previous experiments, IQP-0528 at a concentration of 0.1 μM was added to infected cultures 24, 48, 72 and 96 hours post infection. These cultures were monitored for cell density and viability (not shown), syncytium formation and RT.

In the initial experiment viral replication in the cultures was suppressed when IQP-0528 was added 24 or 48 hours post infection but the cultures had peak infection on day 12 of the ICCA with greater than 1% of the cells in the cultures infected. Cultures with IQP-0528 added 72 hours post infection showed peak infection with greater than 5% of the cells in the culture infected on day 9. Cultures with IQP-0528 added at 96 hours post infection had peak infection greater than 50% of the cells in the culture on day 7. These data are presented in Figure 21A.

RT in the cell free supernatants for the 24 hour and 48 hour cultures was inconsistent. RT was slightly higher than background levels with peak RT on day 5 for the 24-hour culture and day 6 for the 48-hour culture. RT in these cultures began to increase again starting at day 9. RT levels in the 72-hour culture stayed consistent at approximately 5,000 counts per 15 seconds for the duration of the assay with peak RT observed on day 7. The 96-hour culture had peak RT on day 7 of the assay with consistent RT above 10,000 counts per 15 second. These data are presented in Figure 21B.

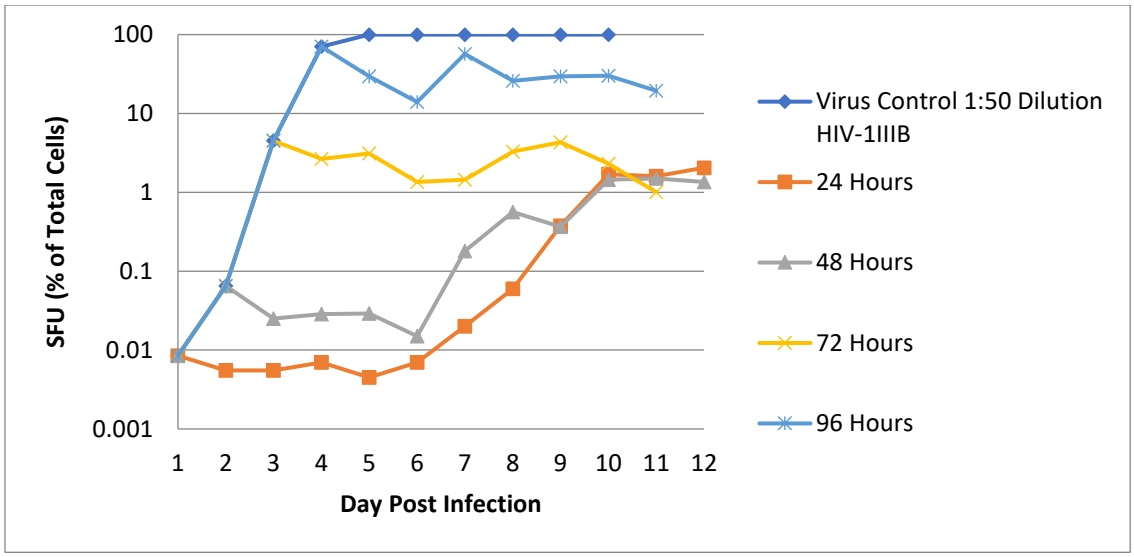


Figure 21A. Percentage of infected cells in ICCA cultures with delayed addition of the sterilizing concentration of IQP-0528, 0.1 µM – Experiment one. IQP-0528 addition 24 hours post infection and 48 hours post infection and had peak infection on day 12. IQP-0528 addition 72 hours post infection had peak infection on day 9. IQP-0528 addition 96 hours post infection showed peak infection on day 7.

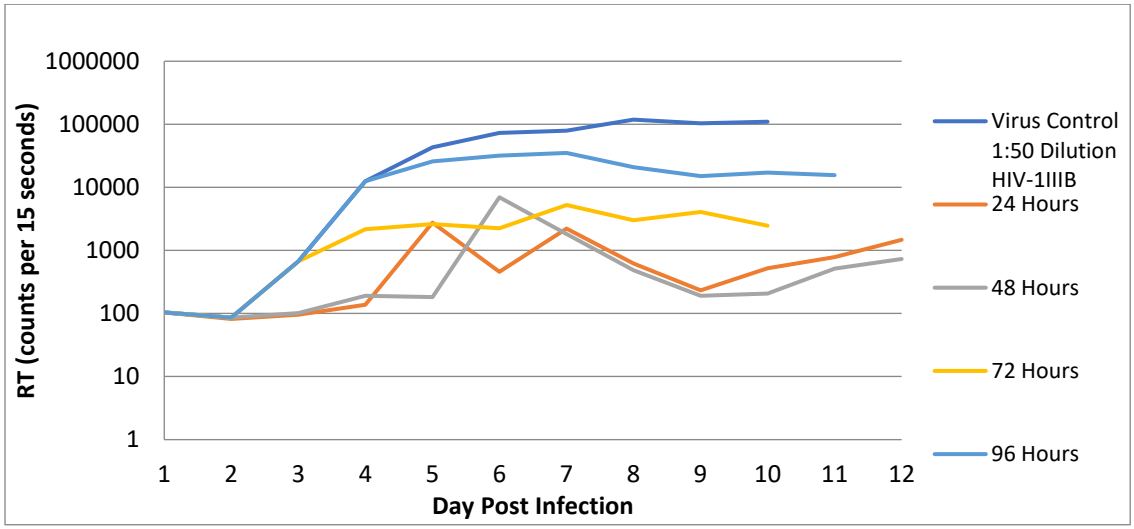


Figure 21B. RT in ICCA cultures with delayed addition of the sterilizing concentration of IQP-0528, 0.1 µM – Experiment one. Cultures with IQP-0528 addition 24 and 48 hours post infection had inconsistent RT. Peak RT was observed on day 5 in the 24-hour culture and on day 6 in the 48-hour culture. Cultures with IQP-0528 added 72 hours post infection had peak RT on day 7. Cultures with IQP-0528 added 96 hours post infection had peak RT on day 7.

In the second experiment, similar results to the first experiment were observed. The addition of 0.1 μM IQP-0528 resulted in a suppression of virus replication in the culture when added 24 to 48 hours post infection but did not result in virus being cleared from the cultures. Peak infection in both the 24 and 48-hour cultures was observed on day 13 with nearly 100% of the cells in the cultures infected. The addition of IQP-0528 at 72 hours post infection influenced the number of infected cells in the cultures, decreasing at day 7 and continuing over time until the end of the assay but not clearing infection. Peak infection occurred on day 6. Peak infection occurred on day 9 in the 96-hour culture. RT These data are presented in Figure 22A.

An increase in RT levels occurred at day 2 and day 3 in the 24 and 48-hour cultures respectively. Peak RT was observed in these cultures on day 14. RT for the 72-hour culture had consistent activity between 1,000 and 10,000 counts per 15 seconds for the duration of the assay, with peak RT observed on day 8. The 96-hour culture also had consistent RT throughout the assay with peak RT on day 8. These data are presented in Figure 22B.

These results suggest that the addition of the sterilizing concentration of IQP-0528 can suppress transmission in cultures when added 24 to 48 hours post infection but is not effective enough to prevent transmission entirely. These data suggest that the addition of a sterilizing concentration of IQP-0528 after infection of cells with HIV would not be sterilizing.

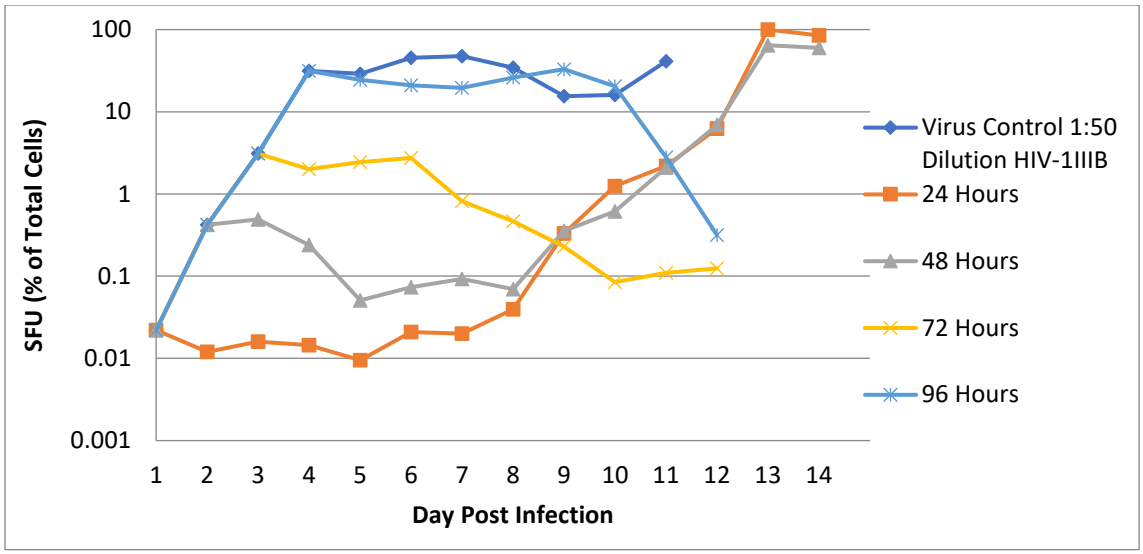


Figure 22A. Percentage of infected cells in ICCA cultures with delayed addition of the sterilizing concentration of IQP-0528, 0.1 µM – Experiment two. Addition of IQP-0528 24 hours and 48 hours post infection had peak infection on day 14. Addition of IQP-0528 72 hours post infection had peak infection on day 6. The decrease in infected cells starting on day 7 can be attributed to the decline in the health of the culture. Addition of IQP-0528 96 hours post infection had peak infection on day 9.

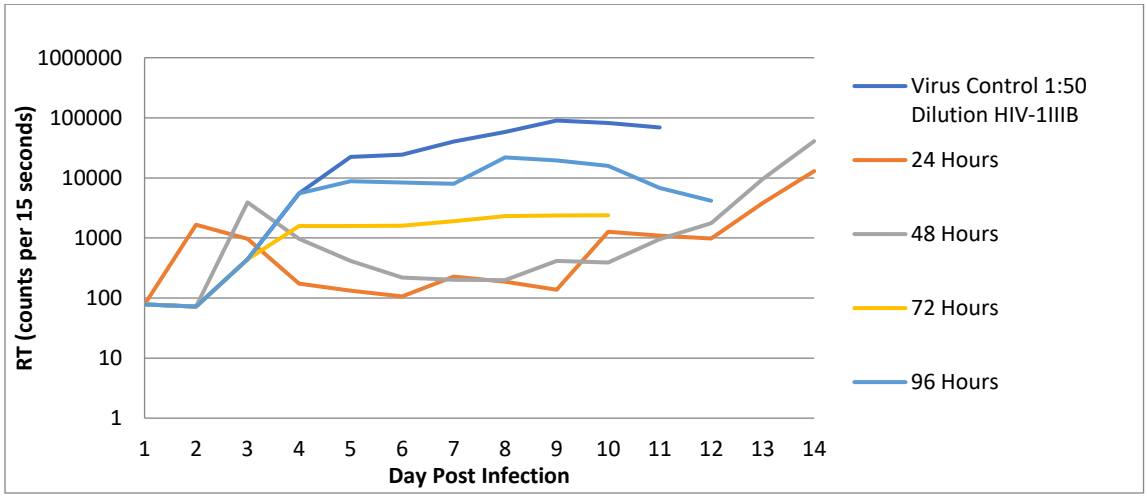


Figure 22B. RT in ICCA cultures with delayed addition of the sterilizing concentration of IQP-0528, 0.1 µM – Experiment Two. Addition of IQP-0528 24 to 48 hours post infection showed an initial increase in RT on day 2 and day 3 followed by a decrease in RT to background levels. RT increases at day 9 and continues to peak activity observed on day 14. IQP-0528 addition 72 hours post infection resulted in an increase of RT from day 1 to day 4, and then had a plateau of RT from day 5 to 10 of the assay, with peak activity observed on day 8. Addition of IQP-0528 96 hours post infection had peak RT on day 8.

Determining the Effect of Delayed Addition of IQP-0528 Following Infection With HIV-1_{IIIB} at Hourly Time Points

The results of the previous experiments indicated that IQP-0528 could not be added after 24 hours and prevent virus replication. To further understand the time of addition of IQP-0528 relative to sterilization of a culture we evaluated a shorter time period for the delay of drug addition to see if we could identify the minimal amount of time drug-addition might be delayed and still yield sterilization. In this experiment IQP-0528 was added at 2, 4, 8, 12, 16, 20 and 24 hours post infection. In these assays the cultures were washed three times prior to the addition of IQP-0528. These cultures were monitored by cell density and viability (not shown), syncytium formation and measurement of cell free supernatant RT.

In the first experiment, the 2 and 4-hour cultures remained below the limit of detection in the ICCA assay. Peak infection of the 8-hour culture occurred on day 14 with 8% of the cells in the culture infected. The 12-hour culture had peak infection on day 14, resulting in greater than 10% of the cells in the culture infected. The 16-hour culture had peak infection on day 14 with 10% of the cells in the culture infected. The 20-hour culture was compromised due to contamination at day 9 of the ICCA. The 24-hour culture had peak infection on day 14 with greater than 10% of the cells of the culture infected. These data are shown in Figure 23A. The 2 and 4-hour cultures were washed to remove IQP-0528 following 12 days of the assay. Both of the cultures became infected at 7 days following the removal of the IQP-0528 as determined by CPE observed in the cultures.

RT in the cell free supernatant was observed in both the 2 and 4-hour cultures prior to compound removal in the ICCA. Both cultures showed peak RT on day 6. The

8, 12, 16 and 24-hour cultures reached peak RT on day 15. These data are presented in Figure 23B.

These data indicate that the addition of the sterilizing concentration of IQP-0528 two hours post infection was not adequate to prevent virus transmission and infection.

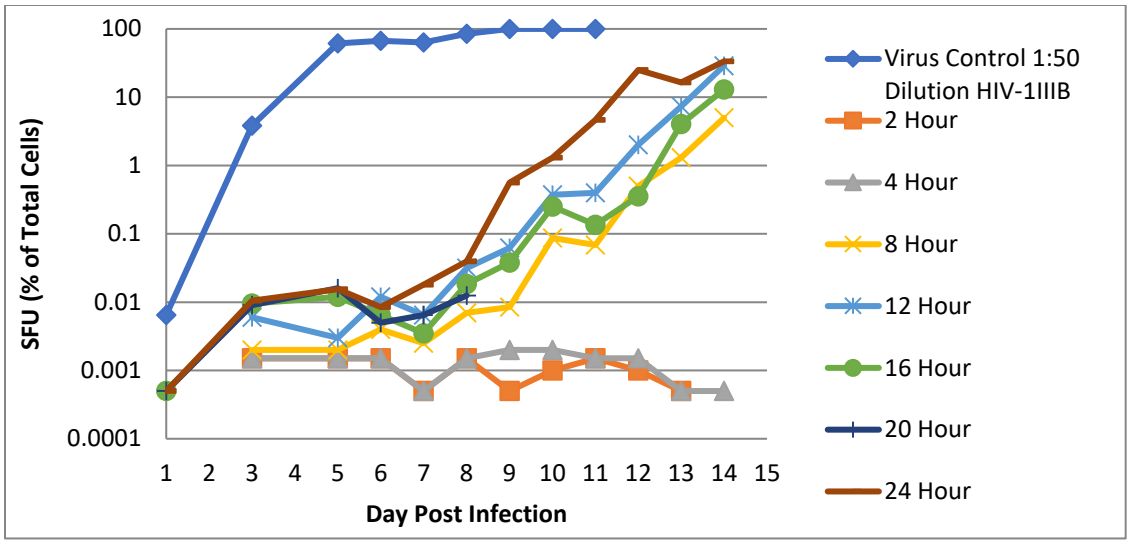


Figure 23A. Percentage of total infected cells in the ICCA cultures with delayed addition of the sterilizing concentration of IQP-0528, 0.1 µM, in hourly increments – Experiment One. Addition of compound greater than four hours post infection showed a delay in infected cells. Addition at 2 and 4 hours post infection remained below the detectable limit.

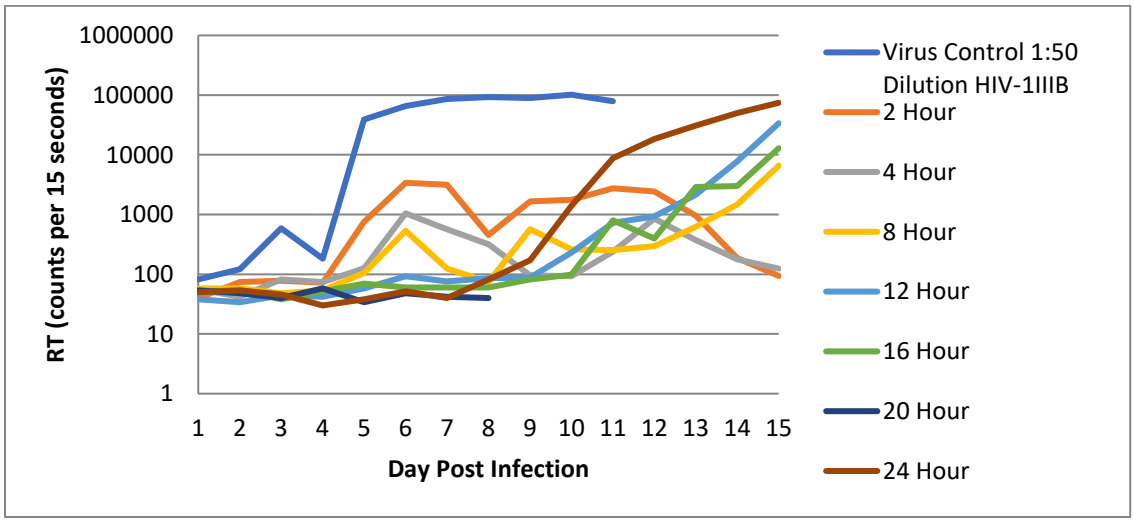


Figure 23B. RT in ICCA cultures with delayed addition of the sterilizing concentration of IQP-0528, 0.1 µM, in hourly increments– Experiment one. There was increased RT in the cultures with 2 hour and 4 hour addition early in the ICCA, which decreased at the end of the assay. Cultures with IQP-0528 addition greater than 4 hours post infection all showed peak RT on day 15.

The second ICCA was performed as above except that IQP-0528 at 0.05 μM, 0.1 μM or 0.2 μM was added at 0, 2, 4, 8 or 12 hours post infection. After 12 days, treated cultures remaining negative for virus infection were continued to be cultured in the presence of IQP-0528. Compound was also removed from treated cultures remaining negative at the end of 12 days to confirm sterilization.

The cultures treated with 0.05 μM IQP-0528 became infected by day 7 of the assay except for the culture where IQP-0528 was added at time 0-hour. Peak infection for the 2 and 4-hour cultures was observed on day 12 with 8% of the cells in the cultures infected. Peak infection also occurred at day 12 in the 8 and 12-hour cultures but with 90% of the cells in the culture infected. These data are presented in Figure 24A. All cultures except the 0-hour culture showed peak RT on day 8, 9 or 12. The 0-hour culture remained at background RT. These data are presented in Figure 24B.

IQP-0528 was removed from the 0-hour culture on day 12 which became infected on day 5 post compound removal (day 17 of the assay) through visual observation of the culture for CPE. The 0-hour culture which was continued in the presence of 0.05 μM IQP-0528 remained negative for virus infection through the end of the assay on day 36.

The 0, 2 and 4-hour cultures when treated with 0.1 μM IQP-0528 remained below the limit of detection for syncytium formation and RT during the 12 days of this ICCA. The 8- and 12-hour cultures had peak infection on day 12, with less than 1% of the cultures infected. Both the 8- and 12-hour cultures had peak RT on day 12. These data are presented in Figure 24C and 24D.

Post compound removal, the 4-hour culture became infected on day 2 post IQP-0528 removal (day 14 of the ICCA). The 4-hour culture with continued IQP-0528

addition became infected on day 6 (day 18 of the ICCA). Post compound removal, the 2-hour culture became infected on day 6 post IQP-0528 removal (day 18 of ICCA). The 2-hour culture continued in the presence of IQP-0528 remained negative for virus infection through the end of the assay on day 36. Post compound removal, the 0-hour time point remained negative for virus infection through the end of the assay. The 0-hour culture continuing in the presence of IQP-0528 also remained negative for virus infection through the end of the assay.

The cultures grown in 0.2 µM IQP-0528 remained below the limit of detection in the SFU assay. These data are shown in Figure 24E. All cultures had no RT above background levels. These data are shown in Figure 24F. Visual observation of CPE following removal of the compound at 4, 8 and 12-hour cultures indicated infection on day 3 (day 15 of the ICCA). The 0 and 2-hour time points remained negative for virus infection through the end of the assay on day 36.

These data show that the addition of IQP-0528 at concentrations below the sterilizing concentration following infection will only prevent transmission of virus if the concentration is maintained. Upon compound removal, viral outgrowth occurs. These data also suggest that IQP-0528 at sub-optimal doses does not prevent infection but suppresses it. These data suggest that if the sterilizing concentration of IQP-0528 is added at the time of infection, the cultures can be sterilized which is supported by the cultures remaining uninfected even when compound is removed. These data also suggest that increasing the concentration of IQP-0528 in the cultures allows for compound to be added at increased time periods post infection. This is supported by adding 0.2 µM IQP-0528 and sterilizing both the 0- and 2-hour cultures.

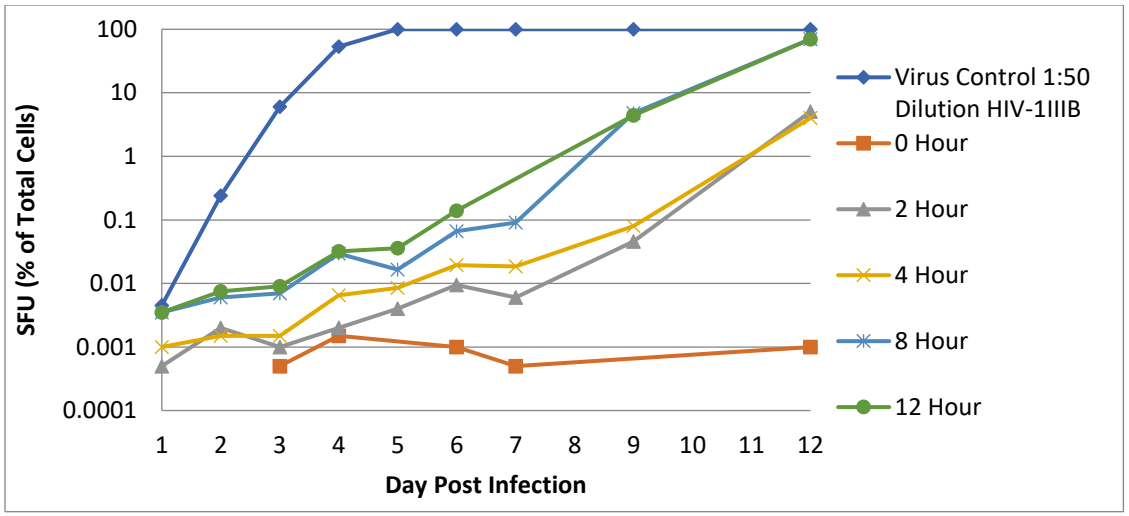


Figure 24A. Percentage of infected cells in cultures infected with HIV-1_{IIIB} with delayed addition of 0.05 µM IQP-0528 in hourly increments – Experiment two. Addition of 0.05 µM IQP-0528 0 hours post infection remained below the limit of detection in the SFU assay. All other cultures at all other concentrations resulted in peak infection on day 12.

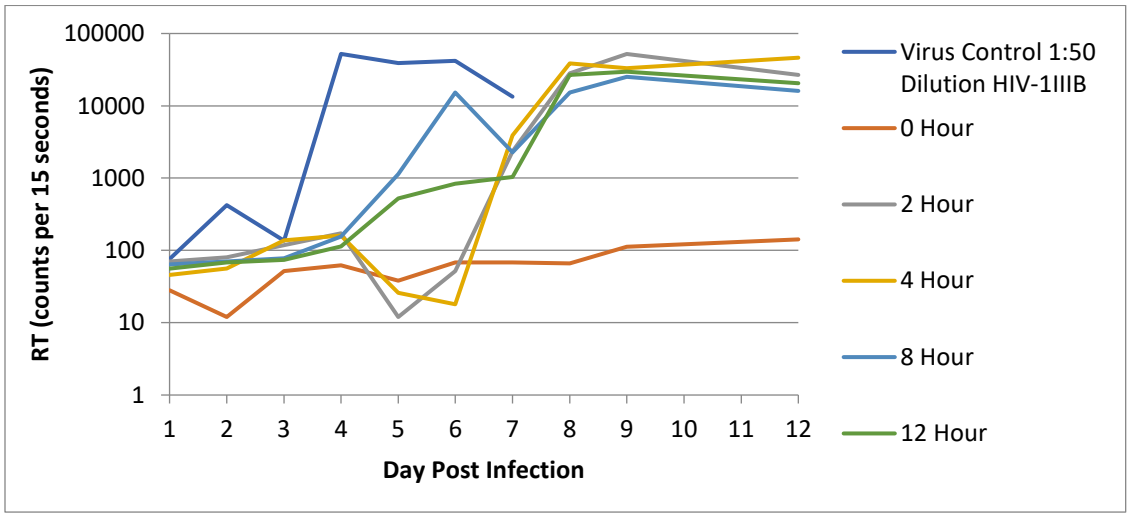


Figure 24B. RT in cultures infected with HIV-1_{IIIB} with delayed addition of 0.05 µM IQP-0528 in hourly increments – Experiment two. Addition of 0.05 µM IQP-0528 greater than 0 hours post infection showed peak RT at day 8, 9 or 12 for all cultures. The addition of IQP-0528 at 0 hours remained at background RT levels.

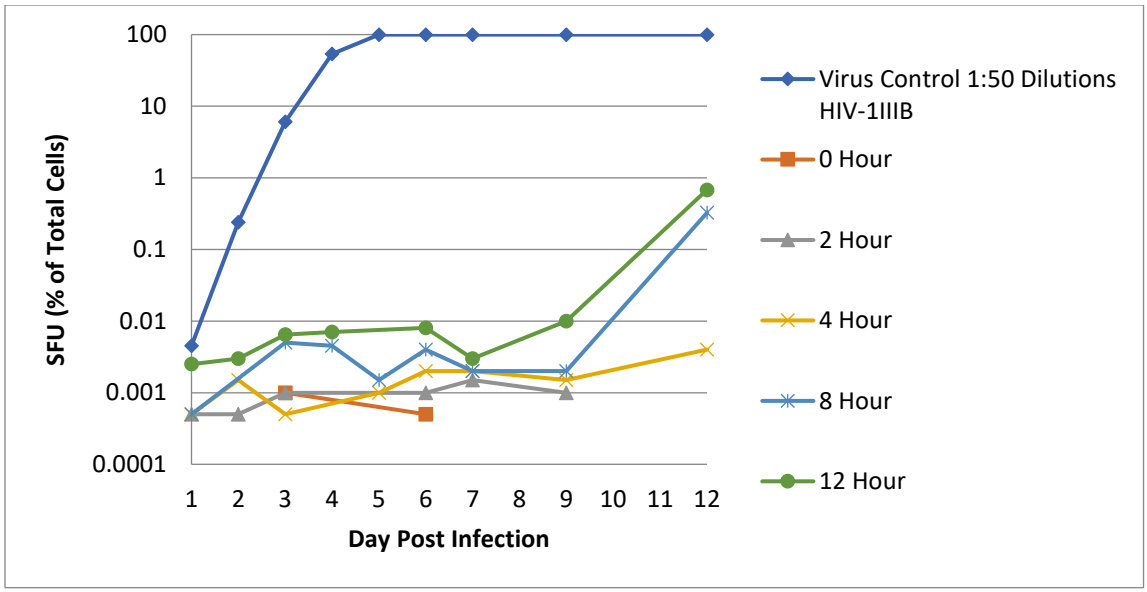


Figure 24C. Percentage of infected cells in cultures infected with HIV-1_{IIIB} and delayed addition of 0.1 µM IQP-0528 in hourly increments – Experiment two. Cultures with addition of compound greater than 4 hours post infection resulted in peak infection on day 12. Cultures with addition of IQP-0528 at or before 4 hours post infection remained below the detectable limit in the SFU assay.

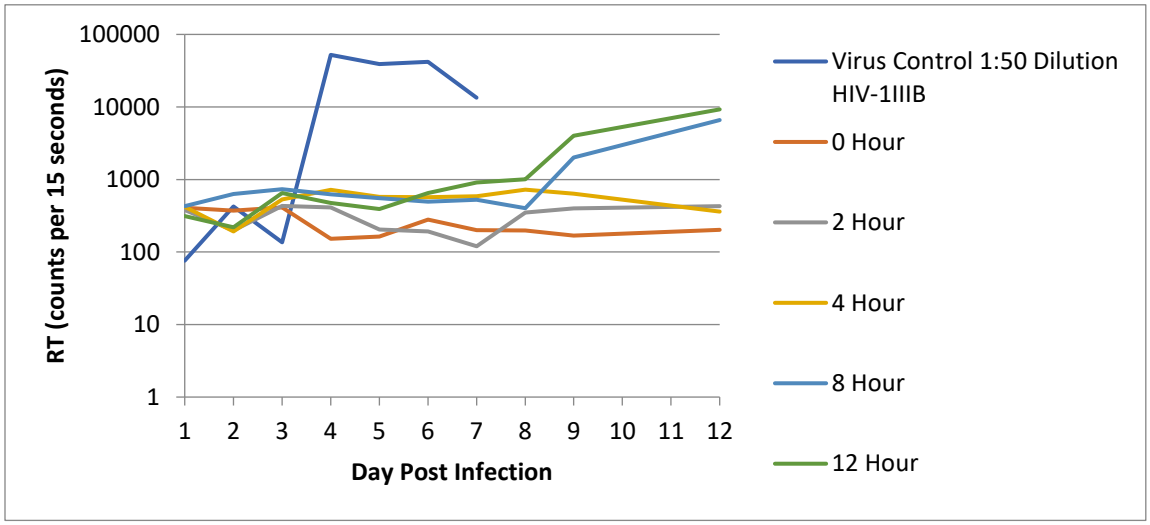


Figure 24D. RT in cultures infected with HIV-1_{IIIB} with delayed addition of 0.1 µM IQP-0528 in hourly increments – Experiment two. Addition of IQP-0528 greater than 4 hours post infection resulted in peak RT observed on day 12. Addition of IQP-0528 at 4 hours post infection resulted in cultures with RT above background but no significant increase throughout the duration of the ICCA. Addition of IQP-0528 at time 0 or 2 hours or post infection resulted in RT at background levels.

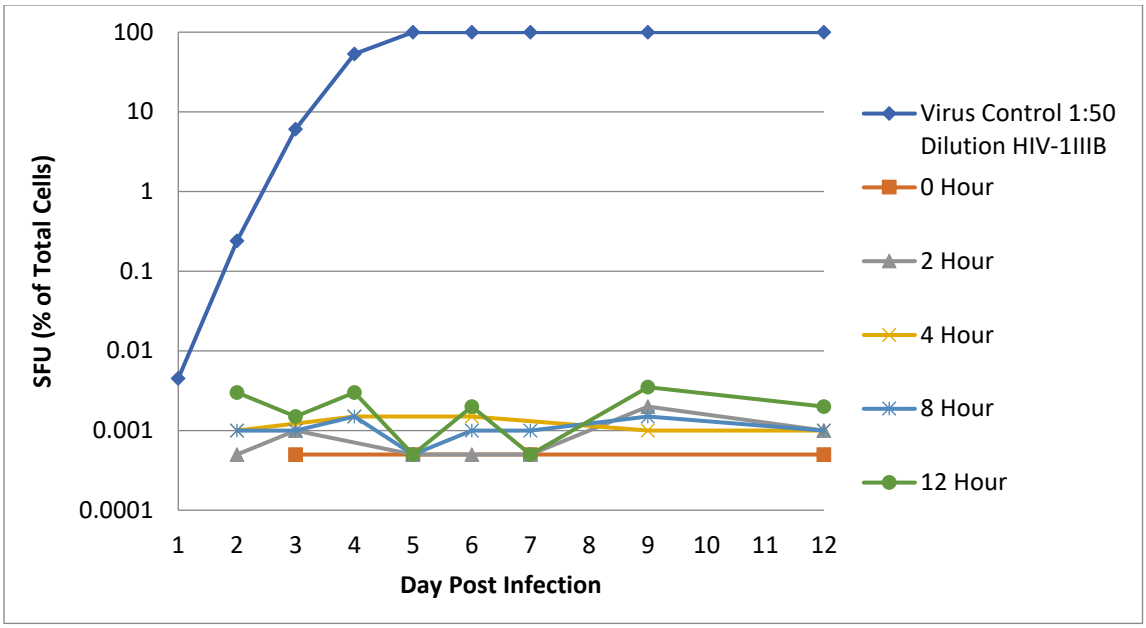


Figure 24E. Percentage of infected cells in cultures infected with HIV-1_{IIIB} and delayed addition of 0.2 µM IQP-0528 in hourly increments – Experiment two. Addition of IQP-0528 at or before 12 hours post infection remained below the detectable limit in the SFU assay.

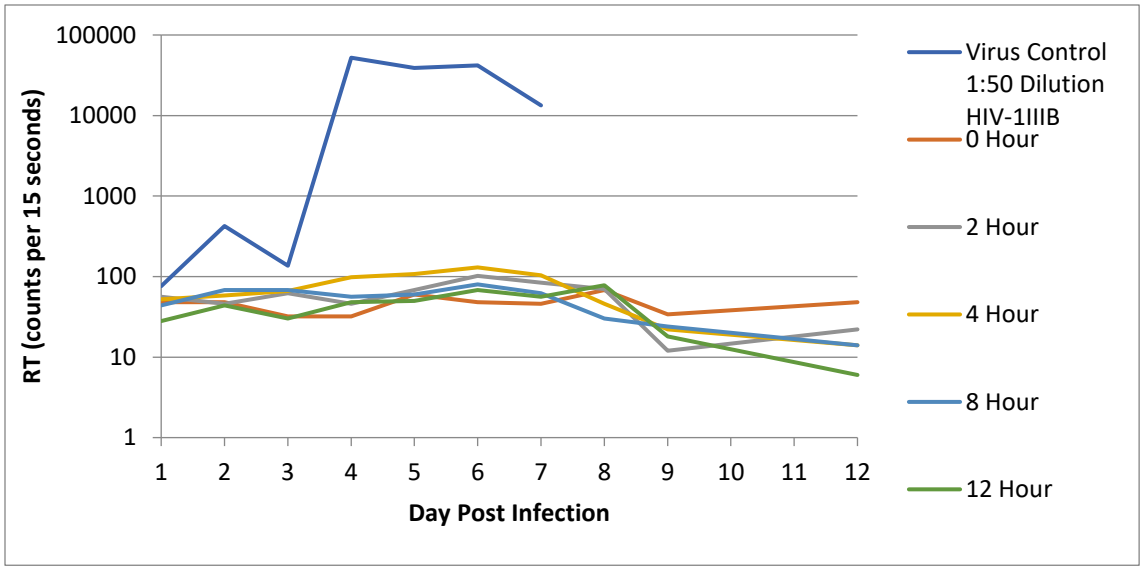


Figure 24F. RT in cultures infected with HIV-1_{IIIB} with delayed addition of 0.2 µM IQP-0528 in hourly increments - Experiment two. Addition of IQP-0528 at or before 12 hours post infection resulted in RT at background levels for all cultures.

Effects of IQP-0528 in the ICCA on HIV-1 Resistant Viruses Critical to the Activity of IQP-0528

HIV-1_{Y181C}

HIV-1_{Y181C} is one of the most commonly selected resistance mutations seen in patients treated long term with most NNRTIs and for this reason we evaluated the effects of IQP-0528 on the transmission and sterilization of HIV-1_{Y181C}. It is important to determine how IQP-0528 will act in the presence of a resistant virus possessing this amino acid change in the RT in order to understand the potential increase in the sterilizing concentration that is necessary to prevent infection with a common resistant virus circulating in the population. The ICCA method was used as described with the following variations: The optimal virus concentration for HIV-1_{Y181C} was determined to be a 1:2 dilution. IQP-0528 is less active against HIV-1 harboring a mutation at amino acid 181 so the concentrations of IQP-0528 were higher than those used against wild type virus and included 2.5 μM, 5 μM, 10 μM and 12.5 μM IQP-0528. The concentrations chosen were based on the relative fold-resistance of viruses with the Y181C amino acid change in the RT when tested against IQP-0528 in acute infection antiviral assays. The ICCA with the Y181C containing virus was performed in duplicate using the same concentrations of IQP-0528 in each replicate assay. These cultures were monitored for cell density and cell viability (not shown), syncytium formation and RT in cell free supernatants.

The virus control in this experiment had peak infection on day 4 with greater than 90% of the cells in the culture infected. The 2.5 μM treated culture had peak infection on day 12 of the assay with greater than 1% of the cells in the culture infected. All

remaining cultures were below the limit of detection in the SFU assay. These data are presented in Figure 25A.

The culture treated with IQP-0528 at 2.5 μM had peak RT on day 6. All other IQP-0528 treated cultures had RT slightly above background levels. These data are presented in Figure 25B.

The culture treated with IQP-0528 at 2.5 μM was confirmed to be infected by PCR performed on day 12. The 5 μM and 10 μM cultures were confirmed to be negative on day 12 by PCR. These data are presented in Figure 25C.

Following removal of the compound, the 5 μM treated culture became infected on day 18 of the assay (6 days after compound removal) and the result was confirmed by PCR. The cultures with the two highest concentrations of compound (10 μM and 12.5 μM) remained negative by visual CPE of the cultures and PCR until the assay ended at 30 days post removal of compound. These data are presented in Figure 25C.

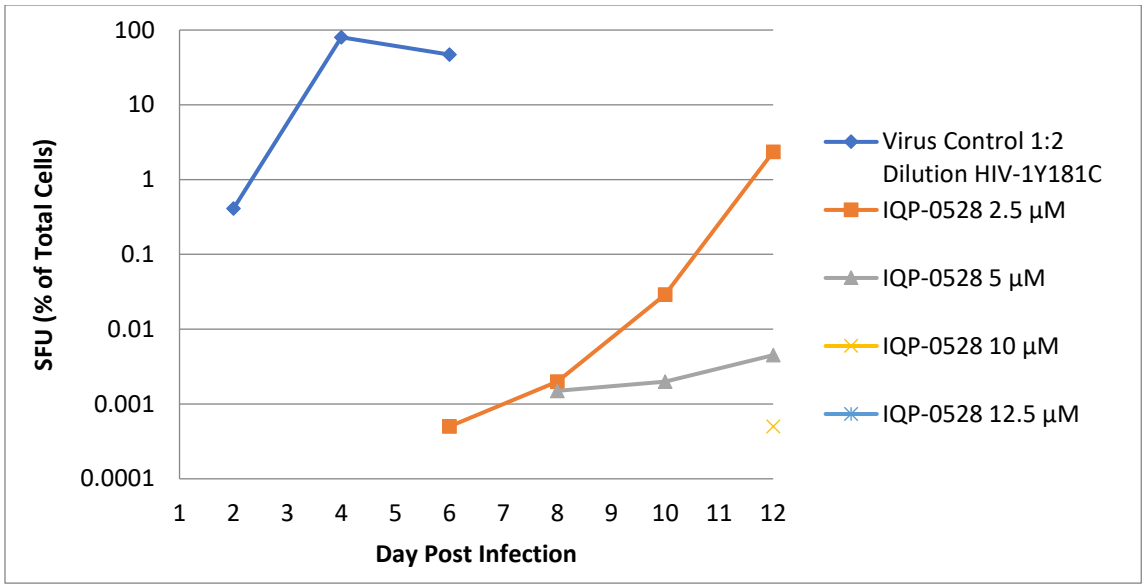


Figure 25A. Percentage of infected cells in the HIV-1_{Y181C} cultures at specified concentrations of IQP-0528 – Experiment one. The 2.5 µM IQP-0528 treated culture had peak infection on day 12. Cultures grown in 5 µM IQP-0528 or greater remained below the detectable limit for infected cells.

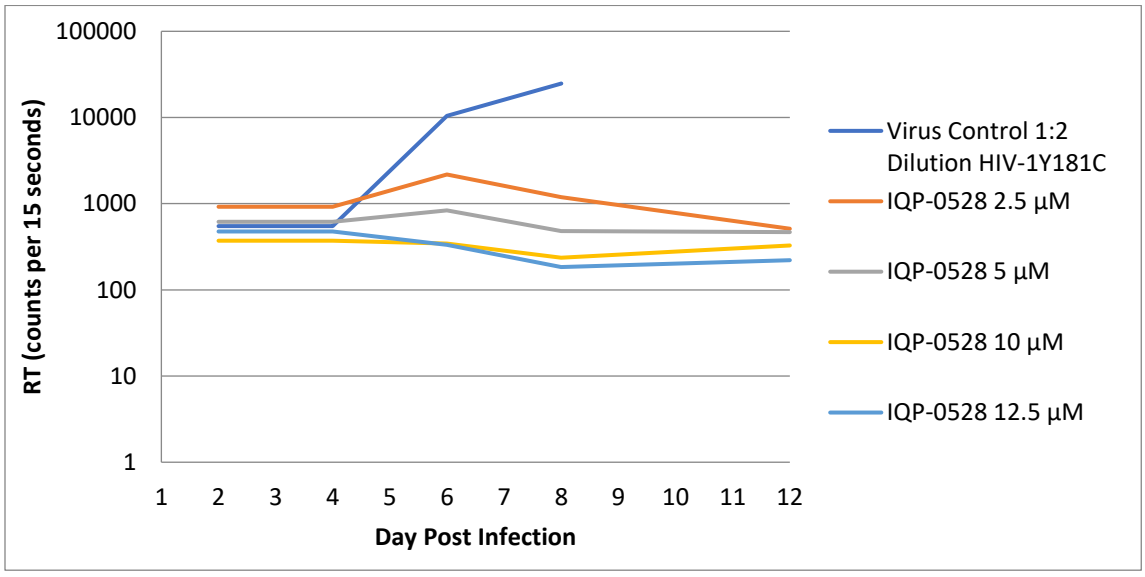


Figure 25B. RT of infected cells in the HIV-1_{Y181C} cultures at specified concentrations of IQP-0528 – Experiment one. The 2.5 µM treated culture had peak RT on day 6. All other cultures had RT slightly above background level.

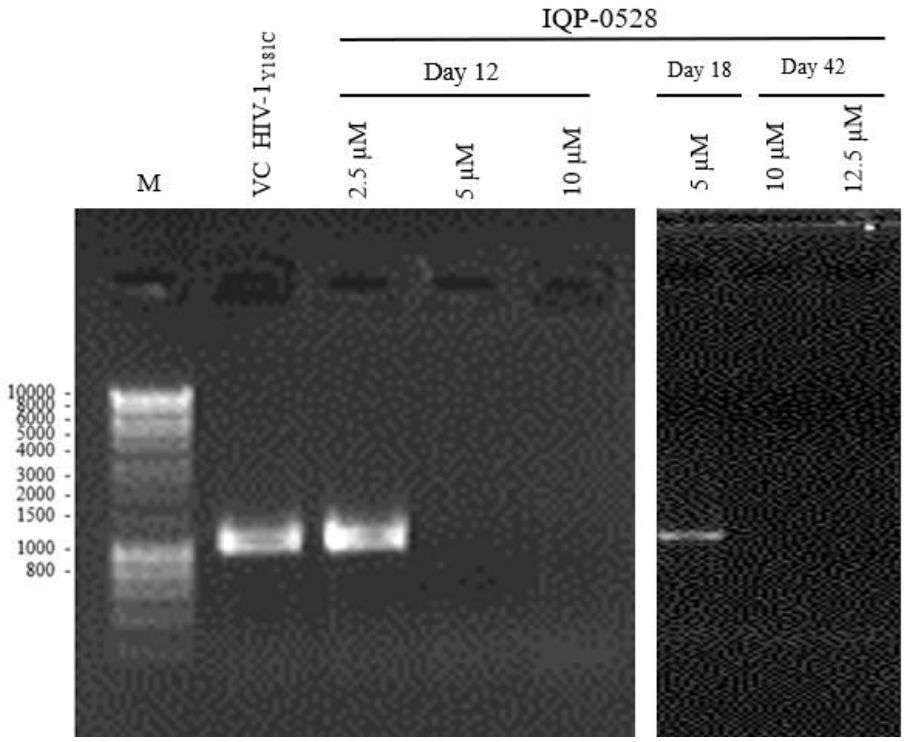


Figure 25C. PCR Results of infected cells in the HIV-1_{Y181C} cultures at specified concentrations of IQP-0528 – Experiment one. Lane 2: Positive PCR results for the virus control on day 12 of the ICCA. Lanes 3-Positive PCR result for the 2.5 µM treated culture on day 12 of the ICCA . Lane 4-5: Negative PCR resuslt for the 5 µM and 10 µM treated cultures on day 12 of the ICCA. Lane 6: Positive PCR result for the 5 µM treated culture on day 18 of the ICCA (day 6 post compound removal). Lanes 7-8: Negative PCR results for the 10 µM and 12.5 µM cultures on day 42 of the ICCA (day 30 post compound removal).

In the second experiment, the virus control had peak infection on day 4. The culture treated with IQP-0528 at 2.5 μM showed syncytia slightly above the limit of detection on days 6 and 8 but returned to below the limit of detection at later timepoints. All other cultures containing IQP-0528 remained below the limit of detection in the SFU assay. These data are shown in Figure 26A.

All cultures had RT slightly above background levels, which was observed in the first experiment. These data are shown in Figure 26B. All treated cultures were shown to be negative for virus infection on day 12 of the ICCA by PCR. These data are presented in Figure 26C.

Following compound removal, the culture treated with IQP-0528 at 2.5 μM became infected on day 16 of the assay (4 days post compound removal) as observed visually for CPE in the culture. The cultures treated with the three highest concentrations (5 μM, 10 μM and 12.5 μM) remained negative as determined by visual observation of the cultures for CPE until completion of the assay at 30 days post removal of compound.

These results suggest that the sterilizing concentration of IQP-0528 is greater than 2.5 μM and potentially greater than 5 μM in the ICCA. This result shows a > 50-fold reduction in the sterilizing capability of IQP-0528 against HIV-1_{Y181C} than against HIV-1_{IIIB}.

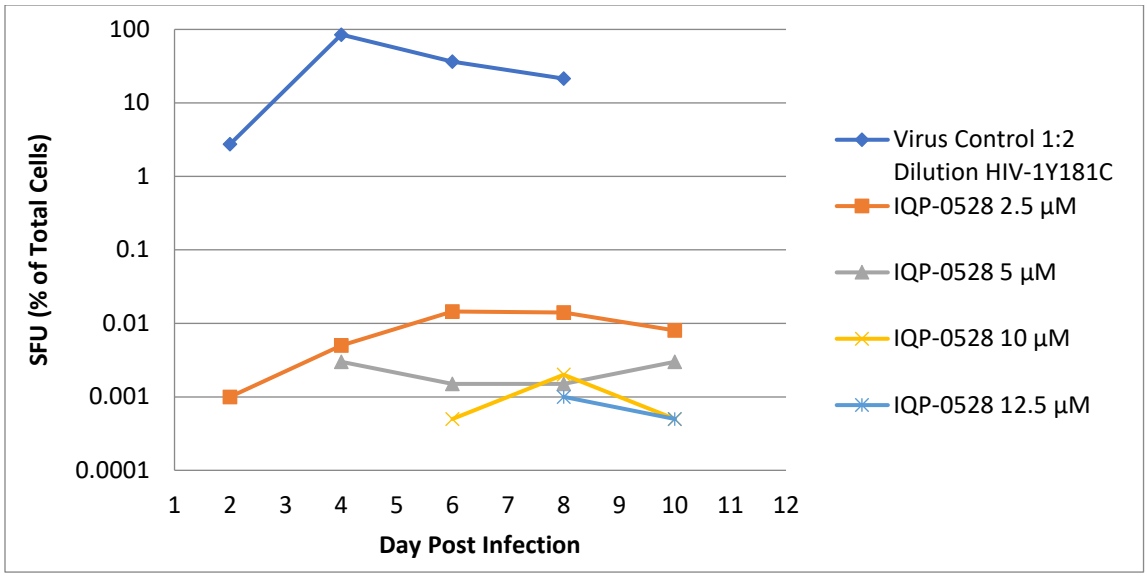


Figure 26A. Percentage of infected cells in the HIV-1_{Y181C} cultures at specified concentrations of IQP-0528 – Experiment two. Two syncytium counts above the limit of detection were observed in the 2.5 µM culture on days 6 and 8. All other cultures remained below the detectable limit in the SFU assay.

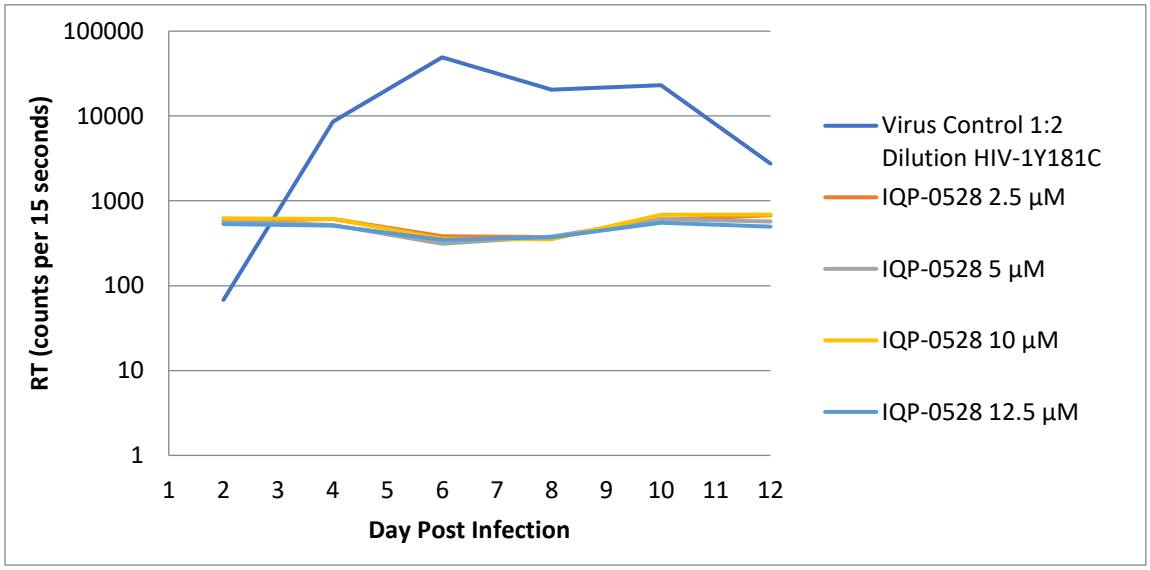


Figure 26B. RT of infected cells in the HIV-1_{Y181C} cultures at specified concentrations of IQP-0528 – Experiment two. All cultures had RT slightly above background levels.

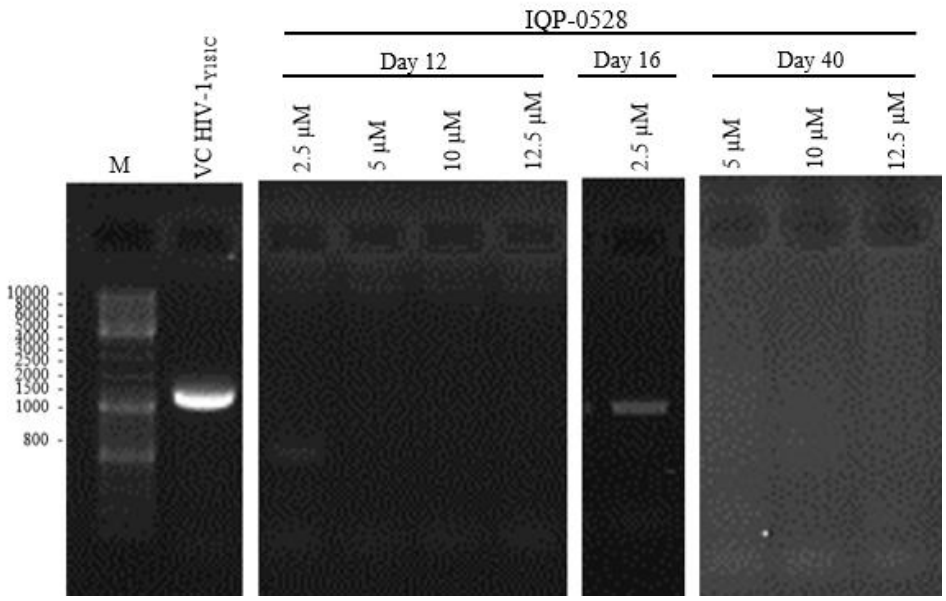


Figure 26C. PCR Results of infected cells in the HIV-1_{Y181C} cultures at specified concentrations of IQP-0528 – Experiment two. Lane 2: Positive PCR result of the virus control on day 12 of the ICCA. Lanes 3-6: Negative PCR results for the 2.5 µM, 5 µM, 10 µM and 12.5 µM treated cultures on day 12 of the ICCA. Lane 7: Positive PCR result for the 2.5 µM treated culture on day 16 of the ICCA (day 4 post compound removal). Lanes 8-10: Negative PCR results for the 5 µM, 10 µM and 12.5 µM treated cultures on day 40 of the ICCA (28 days post compound removal).

HIV-1_{K103N}

HIV-1_{K103N} is another commonly selected resistance mutation seen in patients treated long term with most NNRTIs. It is important to determine how IQP-0528 will act in the presence of a resistant virus possessing this amino acid change in the RT in order to understand the potential increase in the sterilizing concentration that is necessary to prevent infection with a common resistant virus circulating in the population. The effects of IQP-0528 on HIV-1_{K103N} was determined in the ICCA. The ICCA method was performed with the following variations: The optimal virus concentration determined for HIV-1_{K103N} that was used was a 1:2 dilution. IQP-0528 was used at concentrations of 0.1 μM, 1 μM and 10 μM IQP-0528. The concentrations chosen were based on the relative fold-resistance of viruses with the K103N amino acid change in the RT when tested against IQP-0528 in acute infection antiviral assays. The cultures were monitored for cell density and viability (not shown), syncytium formation and RT in cell free supernatant. This assay was performed in duplicate using the same concentrations of IQP-0528.

The HIV-1_{K103N} virus control reached peak infection on day 12 of the assay compared to the day 5 peak usually seen in HIV-1_{IIIB}. The culture treated with IQP-0528 at 0.1 μM had peak infection on day 10 with greater than 50% of the cells in the culture infected. All other cultures remained below the limit of detection in the SFU assay. These data are shown in Figure 27A.

The RT level of the HIV-1_{K103N} virus control and the 0.1 μM IQP-0528 treated culture peaked on day 6 of the assay. The 1 μM and 10 μM cultures had RT comparable to background levels. These data are shown in Figure 27B.

The culture treated with IQP-0528 at 0.1 μM was determined to be infected based on PCR positivity of samples collected on day 12 of the assay. The 1 μM and 10 μM treated cultures were confirmed to be uninfected by PCR. These data are shown in Figure 27C.

Following the removal of compound, the 1 μM and 10 μM cultures remained negative as determined visually for CPE in the cultures through and additional 30 days in culture.

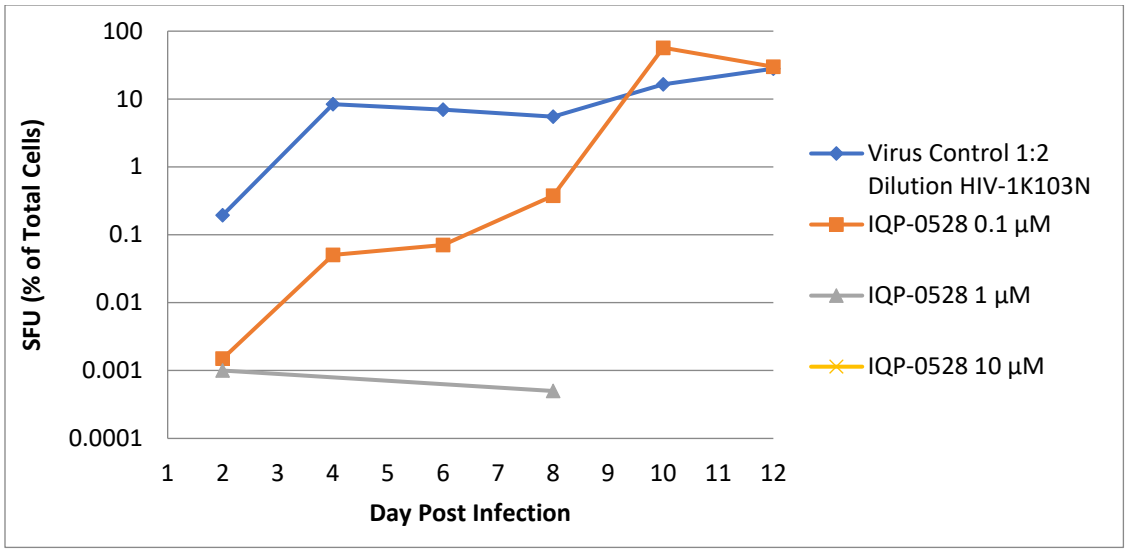


Figure 27A. Percentage of infected cells in the HIV-1_{K103N} cultures at specified concentrations of IQP-0528 – Experiment one. The 0.1 µM treated culture had peak infection on day 10. The 1 µM and 10 µM treated cultures remained below the detectable limit in the SFU assay.

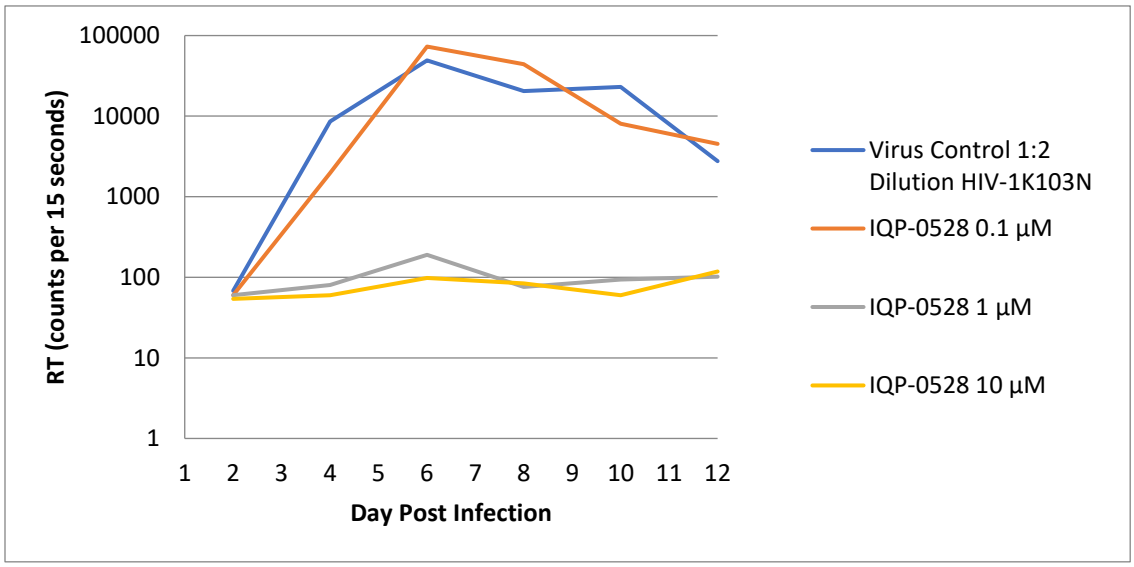


Figure 27B. RT of HIV-1_{K103N} cultures at specified concentrations of IQP-0528 – Experiment one. The 0.1 µM treated culture had peak RT on day 6. The 1 µM and 10 µM cultures showed RT comparable with background levels.

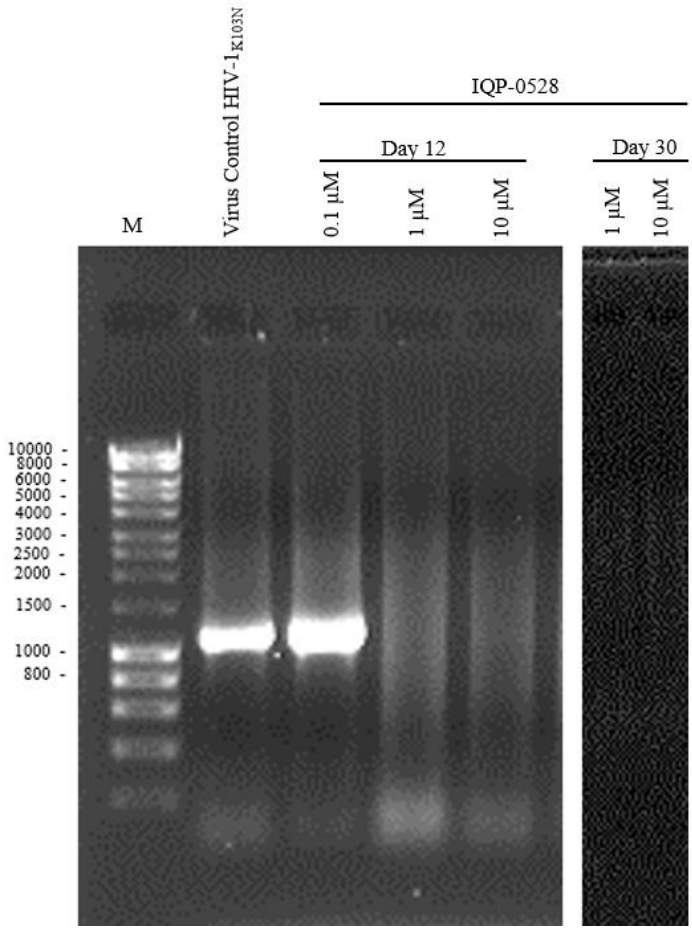


Figure 27C. PCR Results of the HIV-1_{K103N} infected cultures at specified concentrations of IQP-0528 – Experiment one. Lane 2: Positive PCR result of the virus control on day 12 of the ICCA. Lane 3: Positive PCR result for the 0.1 µM treated culture on day 12 of the ICCA. Lanes 4 -5: Negative PCR results of the 1 µM and 10 µM treated cultures on day 12 of the ICCA. Lanes 6 -7: Negative PCR results of the 1 µM and 10 µM treated cultures on day 30 of the ICCA (day 18 post compound removal).

In the second experiment, the virus control demonstrated peak infection on day 12, with greater than 10% of the cells in the culture infected. The culture treated with IQP-0528 at 0.1 μM had peak infection on day 12 of the culture with greater than 1% of the cells in the culture infected. The cultures treated with IQP-0528 at 1 μM and 10 μM remained below the limit of detection in the SFU assay. These data are shown in Figure 28A.

The RT in cell free supernatant for the virus control culture and the culture treated at 0.1 μM were lower than what was seen in the previous experiment. The culture treated with IQP-0528 at 0.1 μM had peak RT on day 12. The culture treated with IQP-0528 at 1 μM showed a spike in RT on day 8 but returned to levels comparable to background on day 10. The culture treated with IQP-0528 at 10 μM had RT comparable to background levels until an increase in activity was seen on day 12. These data are shown in Figure 28B.

The confirmatory PCR performed on the cultures determined all cultures to be negative for infection on day 12 of the assay. Following compound removal from all cultures, the 0.1 μM treated culture became infected as determined visually for CPE in the culture and confirmed by PCR on day 4 following compound removal (day 16 of the ICCA). The cultures treated with IQP-0528 at 1 μM and 10 μM were uninfected based on the absence of CPE in the cultures through 30 days. These data are presented in Figure 28C.

These results suggest that the sterilizing concentration of IQP-0528 in the presence of HIV-1_{K103N} is greater than 1 μM. This is 10-fold lower activity against HIV-1_{K103N} compared to HIV-1_{IIIB}.

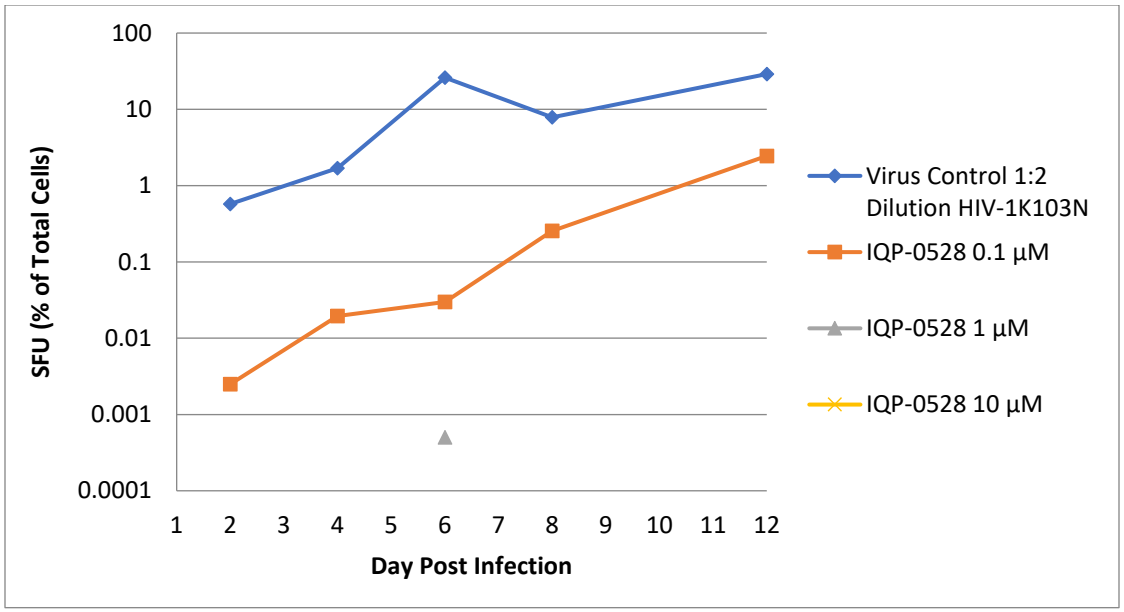


Figure 28A. Percentage of infected cells in the HIV-1_{K103N} cultures at specified concentrations of IQP-0528 – Experiment two. The 0.1 µM treated culture had peak activity on day 12. The 1 µM and 10 µM treated cultures remained below the limit of detections in the SFU assay.

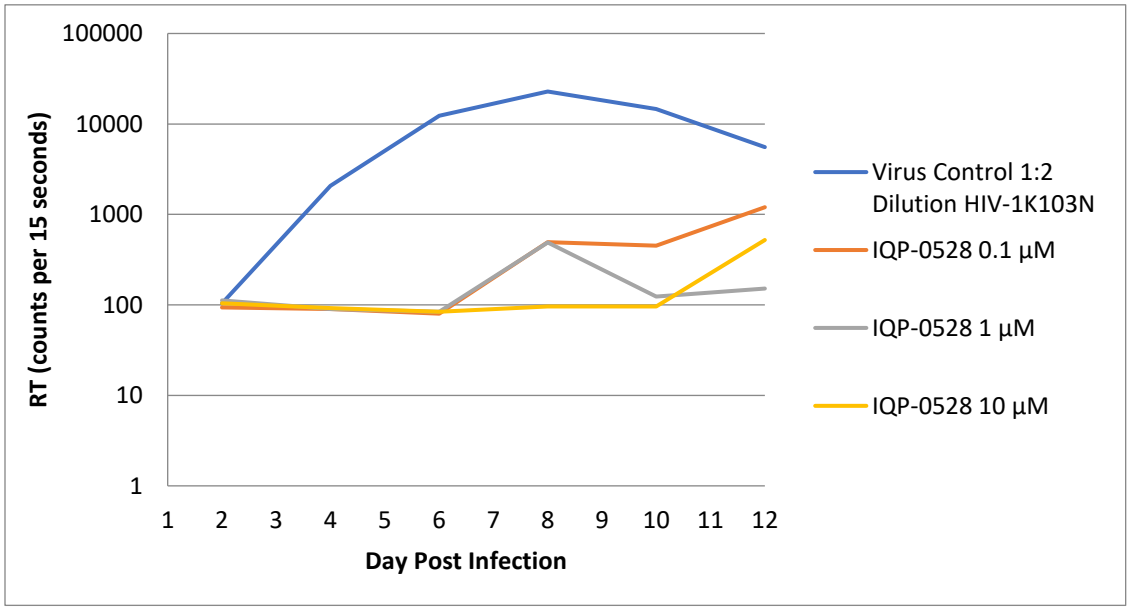


Figure 28B. RT of infected cells in the HIV-1_{K103N} cultures at specified concentrations of IQP-0528 – Experiment two. The 0.1 µM treated culture had peak RT on day 12. The 1 µM and 10 µM treated cultures had a slight increase in RT above background levels through the 12 days of the assay, but no consistent increase in activity.

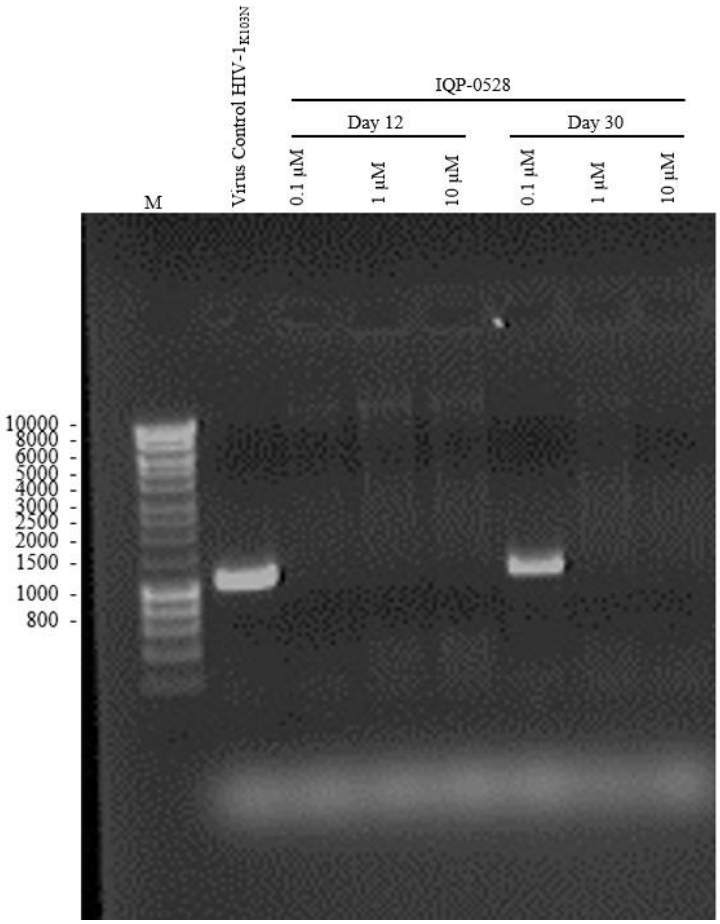


Figure 28C. PCR results of infected cells in the HIV-1_{K103N} cultures at specified concentrations of IQP-0528 – Experiment two. Lane 2: Positive PCR result for the virus control on day 12 of the ICCA. Lanes 3-5: Negative PCR results of the 0.1 µM, 1 µM and 10 µM treated cultures on day 12 of the ICCA. Lane 6: Positive PCR results of the 0.1 µM treated culture on day 16 of the ICCA (day 4 post compound removal). Lanes 7-8: Negative PCR results for the 1 µM and 10 µM cultures on day 31 of the ICCA (day 19 post compound removal).

A17

HIV-1_{A17} is a resistant virus containing both the Y181C and K103N mutations, making it highly resistant to treatment with NNRTIs. It is important to determine the effect of this double mutation in the viral RT on the sterilizing concentration of IQP-0528 since like the viruses with the Y181C and the K103N amino acid changes, the double mutant virus also is circulating in the population of HIV viruses due to previous use of NNRTIs as therapeutic agents. The effects of IQP-0528 on HIV-1_{A17} was determined in the ICCA. The ICCA method was performed with the following variations: The optimal virus concentration determined for HIV-1_{A17} was a 1:5 dilution. In the first experiment, IQP-0528 was used at concentrations of; 0.02 μM, 0.1 μM, 0.5 μM, 2.5 μM and 12.5 μM IQP-0528 and in the second experiment the concentrations used were; 0.46 μM, 1.2 μM, 2.9 μM, 7.2 μM and 17.9 μM. The concentrations chosen for evaluation were defined based on the activity of IQP-0528 in acute infection antiviral assays against viruses with the double mutation in the RT. The cultures were monitored for cell density and viability (not shown), syncytium formation and RT in the cell free supernatant.

All cultures, excluding the culture treated with IQP-0528 at 12.5 μM, had peak infection on day 4 of the assay. The culture treated at 12.5 μM had a peak of infection on day 10 of the assay, with greater than 1% of the cells in the culture infected. These data are shown in Figure 29A.

RT in the HIV-1_{A17} cultures was generally low in all cultures evaluated. Peak RT was observed on day 6 for the virus control and the 0.02 μM and 0.1 μM treated cultures. RT in the remaining cultures was comparable to background levels. These data are shown in Figure 29B.

All cultures were confirmed by PCR to be infected on day 12 of the assay. The results are shown in Figure 29C.

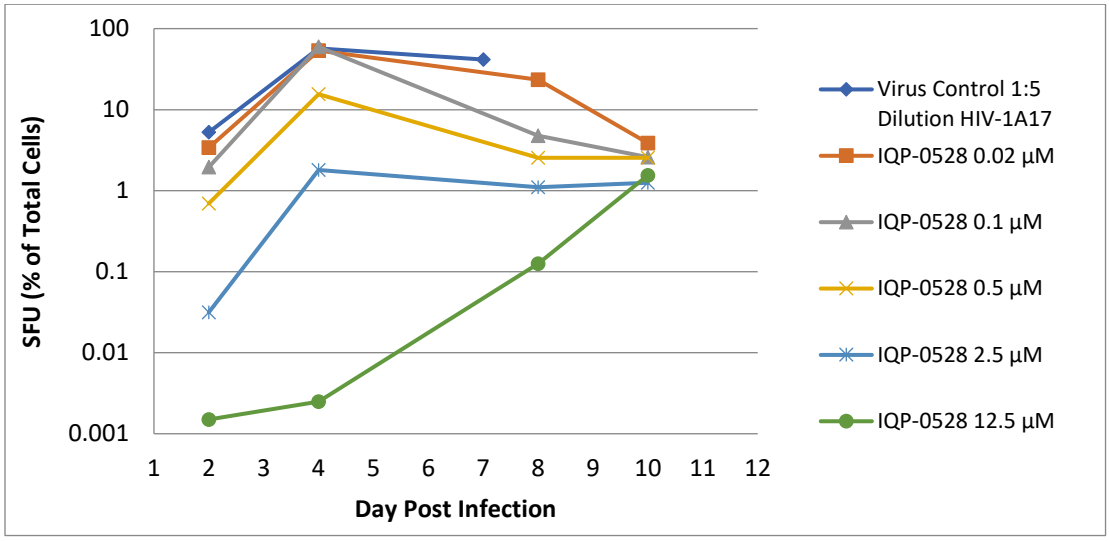


Figure 29A. Percentage of infected cells in the HIV-1_{A17} cultures at specified concentrations of IQP-0528 – Experiment one. Peak infection was observed on day 4 in cultures treated with IQP-0528 concentrations at or below 2.5 µM. The 12.5 µM treated cultures had a peak infection on day 10.

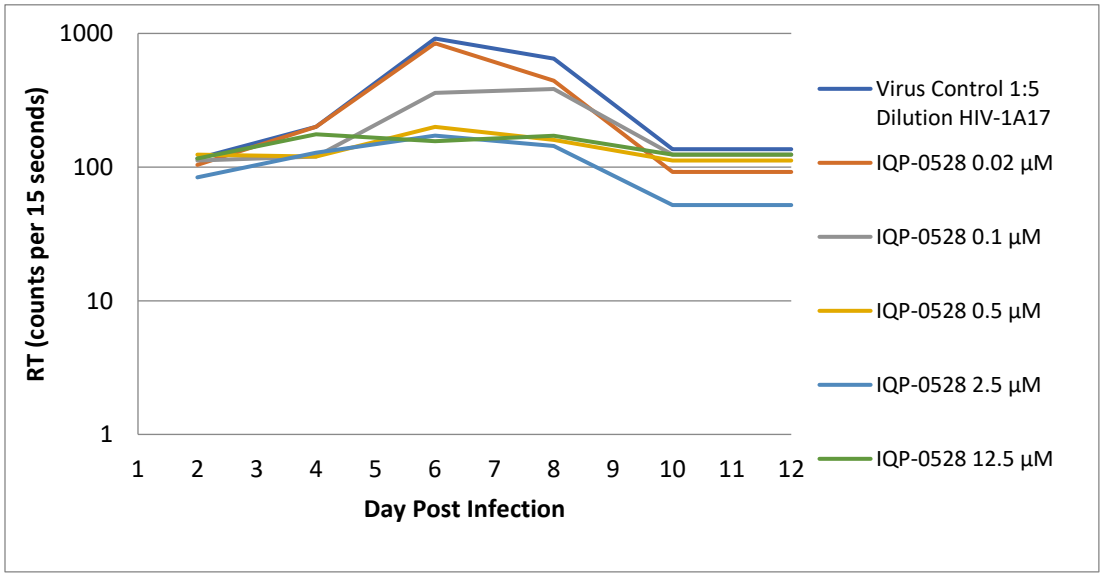


Figure 29B. RT in the HIV-1_{A17} cultures at specified concentrations of IQP-0528 – Experiment one. The virus control and cultures treated with 0.02 µM and 0.1 µM cultures had peak RT on day 6. The remaining cultures had RT comparable to background levels.

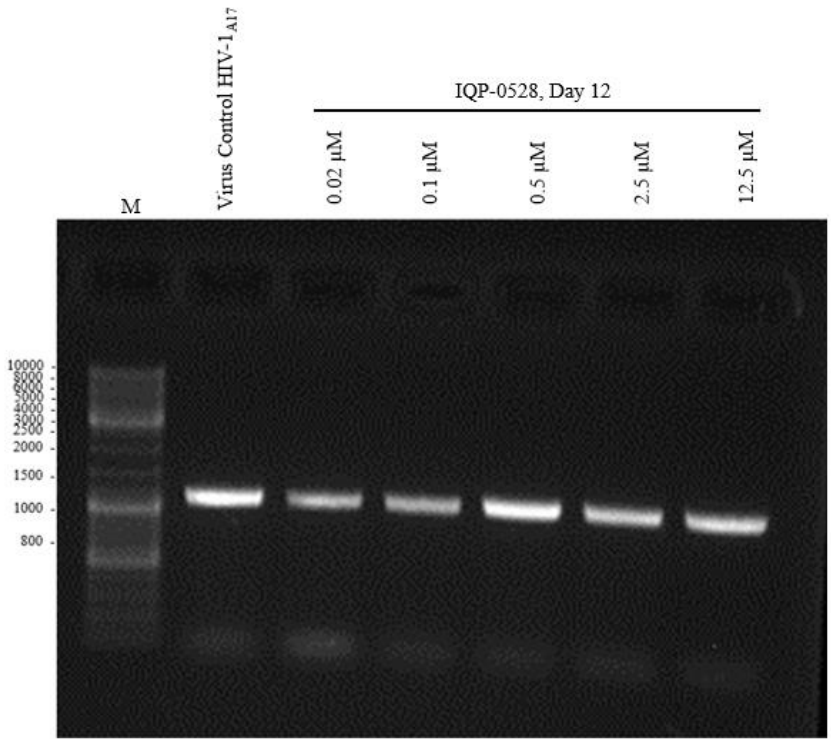


Figure 29C. PCR results of infected cells in the HIV-1_{A17} cultures at specified concentrations of IQP-0528 – Experiment one. Lane 2: Positive PCR results of the virus control on day 12 of the ICCA. Lanes 3-7: Positive PCR results of the 0.02, 0.1, 0.5, 2.5 and 12.5 µM treated cultures on day 12 of the ICCA.

In the second experiment, all cultures treated with IQP-0528 at concentrations at or below 2.9 μM had a peak of infection on day 4. The cultures treated with IQP-0528 at 7.2 μM and 17.9 μM had peak infection on day 10. These data are shown in Figure 30A.

The virus control culture had peak RT on day 8. The culture treated at 0.046 μM had a large increase in RT on day 10 of the ICCA, but RT returned to background levels on day 12. All other cultures had RT levels comparable to background levels. Data are shown in Figure 30B.

Infection of the cultures was confirmed on the day 12 samples using PCR. These data are shown in Figure 30C.

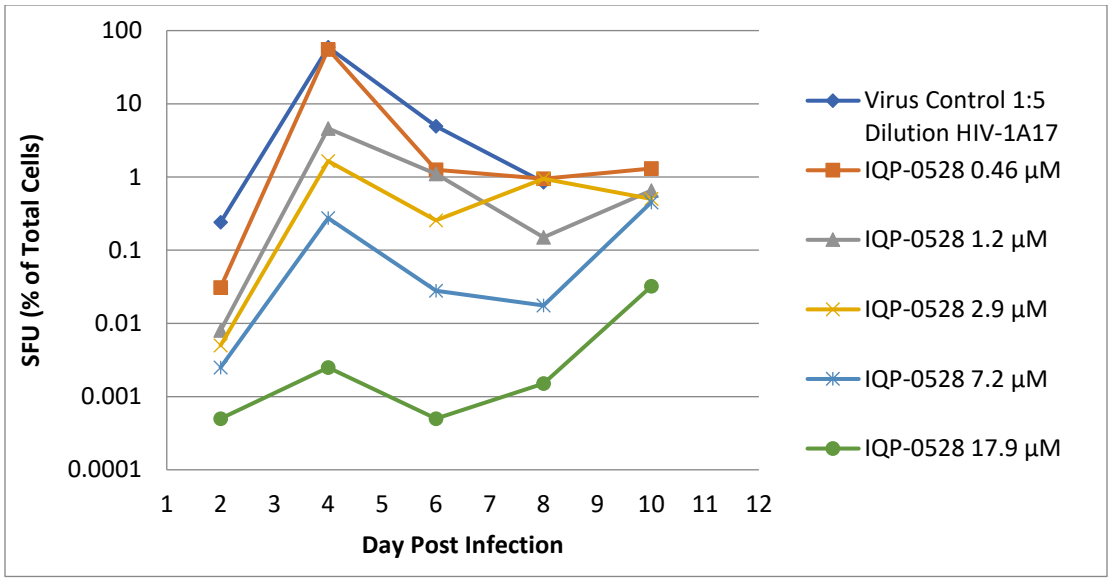


Figure 30A. Percentage of infected cells in the HIV-1_{A17} cultures at specified concentrations of IQP-0528 – Experiment two. Peak infection occurred on day 4 in the cultures with IQP-0528 concentrations at 2.9 µM or lower. Peak infection occurred on day 10 in the cultures treated with 7.9 µM and 17.9 µM.

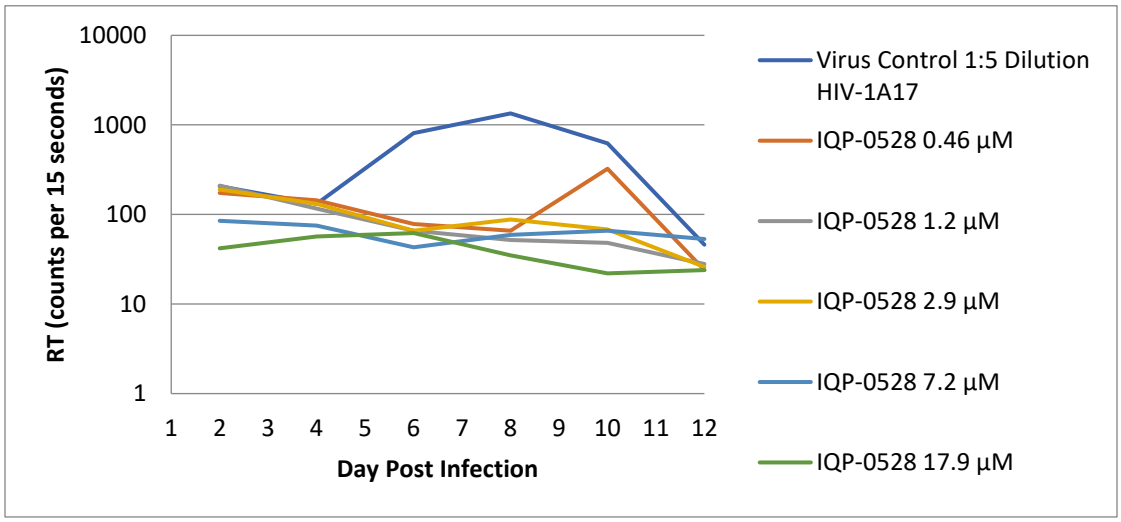


Figure 30B. RT in the HIV-1_{A17} cultures at specified concentrations of IQP-0528 – Experiment two. The virus control culture had peak RT on day 8. The 0.046 µM treated culture showed an RT spike on day 10, but returned to background RT levels on day 12. All other cultures had RT comparable to background levels.

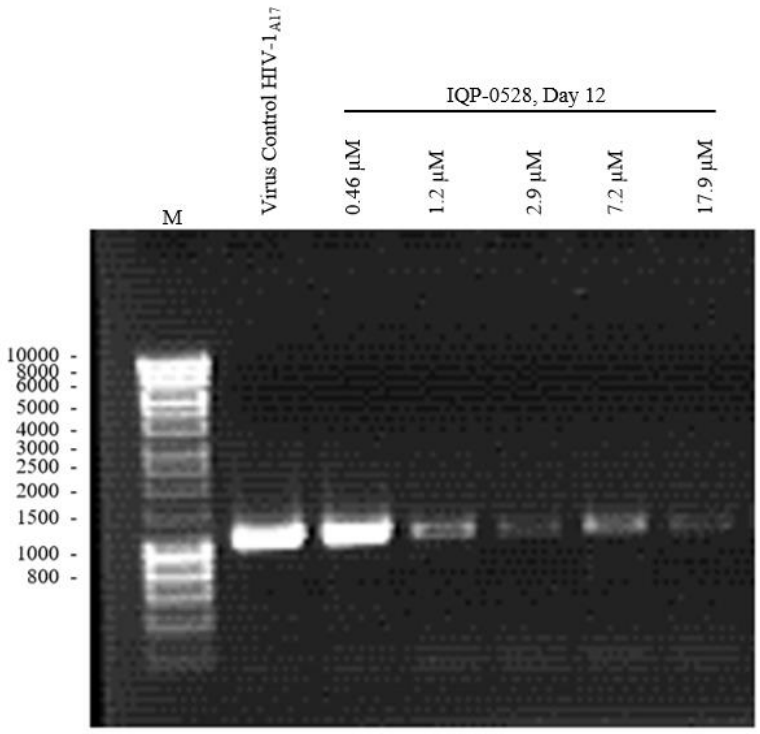


Figure 30C. PCR results of infected cells in the HIV-1_{A17} cultures at specified concentrations of IQP-0528 – Experiment two. Lane 2: Positive PCR results for the virus control culture on day 12 of the ICCA. Lanes 3-7: Positive PCR results for the 0.46 µM, 1.2 µM, 2.9 µM, 7.2 µM and 17.9 µM cultures on day 12 of the ICCA.

Determination of Sterilizing Concentrations of Dapivirine in the ICCA with HIV-1

HIV-1_{IIIB}

Dapivirine is a nonnucleoside RT inhibitor that is approved for use as a therapeutic agent for HIV treatment and has been widely evaluated for use as a prevention agent as a component of gels and intravaginal rings. In light of its developmental status and similarities to IQP-0528 we wanted to evaluate the activity of dapivirine in the ICCA as a direct comparator for microbicide development potential. The sterilizing concentration of dapivirine was determined in the ICCA in replicate experiments. The initial ICCA wit dapivirine was performed according to the methodology described with the following variations: HIV-1_{IIIB} at a dilution of 1:50 was used to infect the CEM-SS cells and dapivirine was evaluated at 0.001 μM, 0.01 μM, 0.1 μM, and 1 μM based on its efficacy in the CPE assay against HIV-1_{IIIB}. The second ICCA was also performed with the same dilution of HIV-1_{IIIB}, but with a narrower range of concentrations of dapivirine that included 0.01 μM, 0.015 μM, 0.02 μM, 0.025 μM and 0.05 μM. The cultures were monitored for cell density and viability (not shown), syncytium formation and RT in cell free supernatant.

Peak infection for the culture treated with dapivirine at 0.001 μM occurred on day 8. All other concentrations of dapivirine remained below the limit of detection in the SFU assay. These data are shown in Figure 31A.

Peak RT was observed on day 10 in the 0.001 μM treated culture. RT was observed in the 0.01 μM treated culture with peak RT on day 12. All other concentrations of dapivirine had RT slightly above background levels but with no discernable peak day. These data are shown in Figure 31B.

Evaluation by PCR determined that the only culture to be infected on day 12 of the ICCA besides the virus control culture was the 0.001 µM treated culture. These data are presented in Figure 31C.

Following compound removal, the culture treated with dapivirine at 0.01 µM became infected based on the appearance of CPE in the culture, which was confirmed by PCR. All other cultures remained uninfected through 40 days.

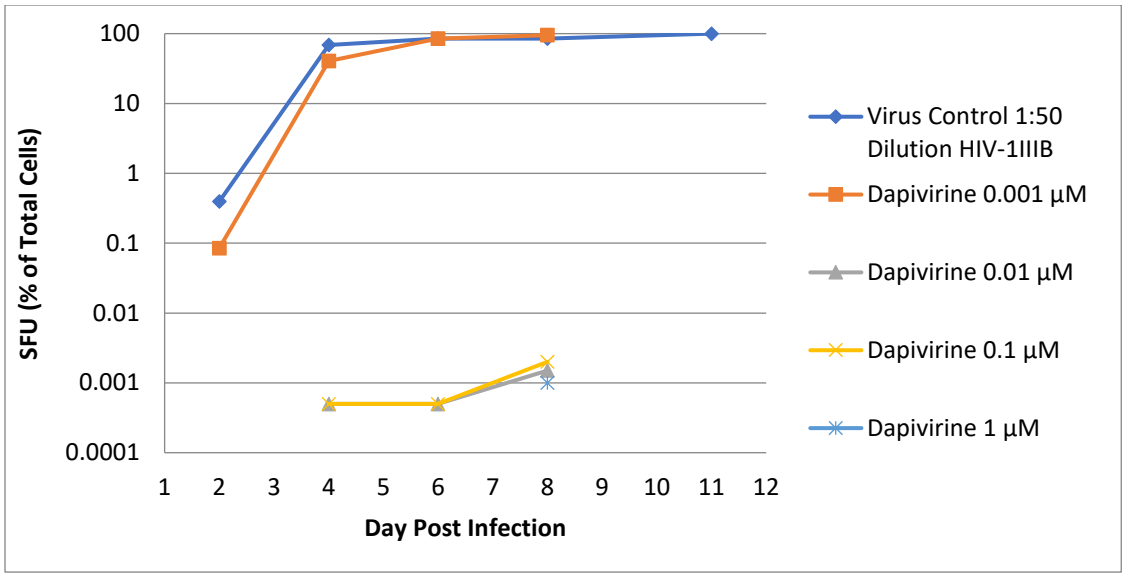


Figure 31A. Percentage of infected cells in the HIV-1_{IIIB} cultures at specified concentrations of dapivirine – Experiment one. The 0.001 µM treated culture had peak infection on day 8. All other cultures remained below the detectable limit for the SFU assay.

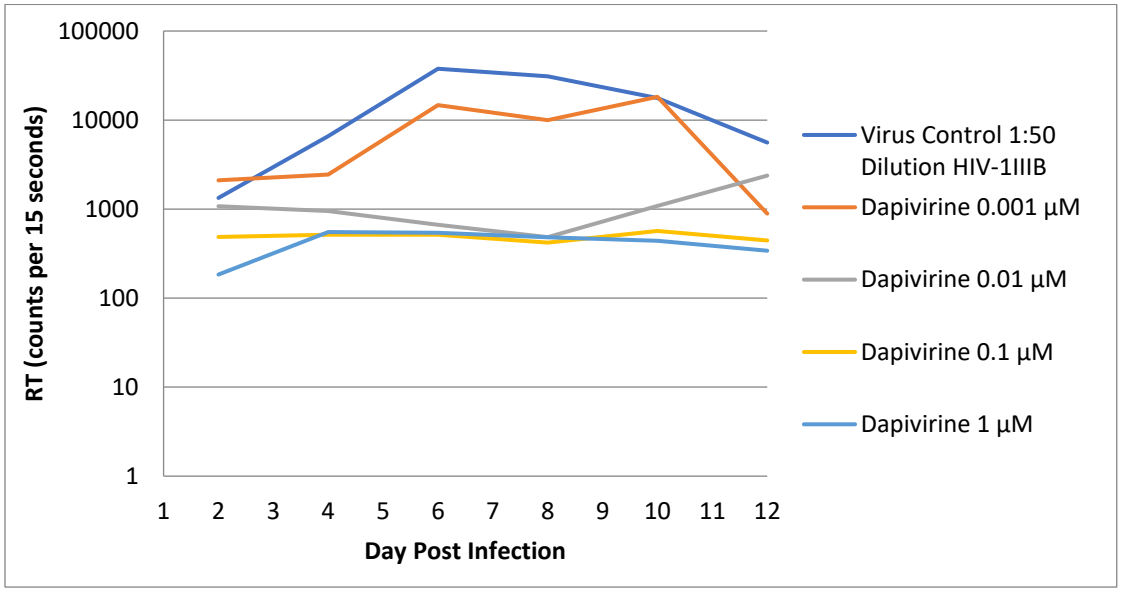


Figure 31B. RT in the HIV-1_{IIIB} cultures at specified concentrations of dapivirine - Experiment one. Peak RT was observed on day 10 in the 0.001 µM treated culture. The 0.01 µM treated culture had peak RT on day 12. All other cultures with concentrations of dapivirine had RT above background levels but with no descernable peak.

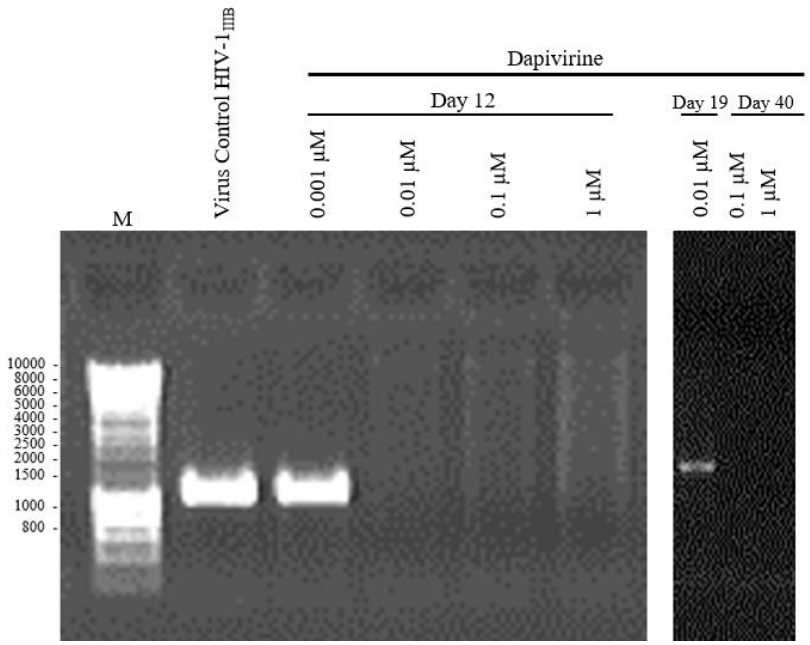


Figure 31C. PCR results of infected cells in the HIV-1_{IIIB} cultures at specified concentrations of dapivirine – Experiment one. Lane 2: Positive PCR result for the virus control on day 12 of the ICCA. Lane 3: Positive PCR result for the 0.001 µM treated culture on day 12 of the ICCA. Lanes 4- 6: Negative PCR results for the 0.01 µM, 0.1 µM and 1 µM treated cultures on day 12 of the ICCA. Lane 7: Positive PCR result for the 0.01 µM treated culture on day 19 of the ICCA (day 7 post compound removal). Lanes 8- 9: Negative PCR results for the 0.1 µM and 1 µM cultures on day 40 of the ICCA (28 days post compound removal).

In the second experiment all cultures remained below the detectable limit in the SFU assay (Figure 32A) and had RT comparable to background levels (Figure 32B). All samples evaluated form the cultures on day 12 of the ICCA were confirmed by PCR to be negative for infection.

Following compound removal, the cultures treated with dapivirine at 0.01μM, 0.015 μM and 0.02 μM became infected on day 25 of the assay (day 13 post compound removal) based on visual observation of CPE in the cultures and by PCR. The cultures treated with dapivirine at 0.025 μM and 0.05 μM remained uninfected through 40 days. These data are presented in Figure 32C.

These results suggest that the sterilizing concentration of dapivirine in the ICCA with HIV-1_{IIIB} is between 0.02 μM and 0.025 μM.

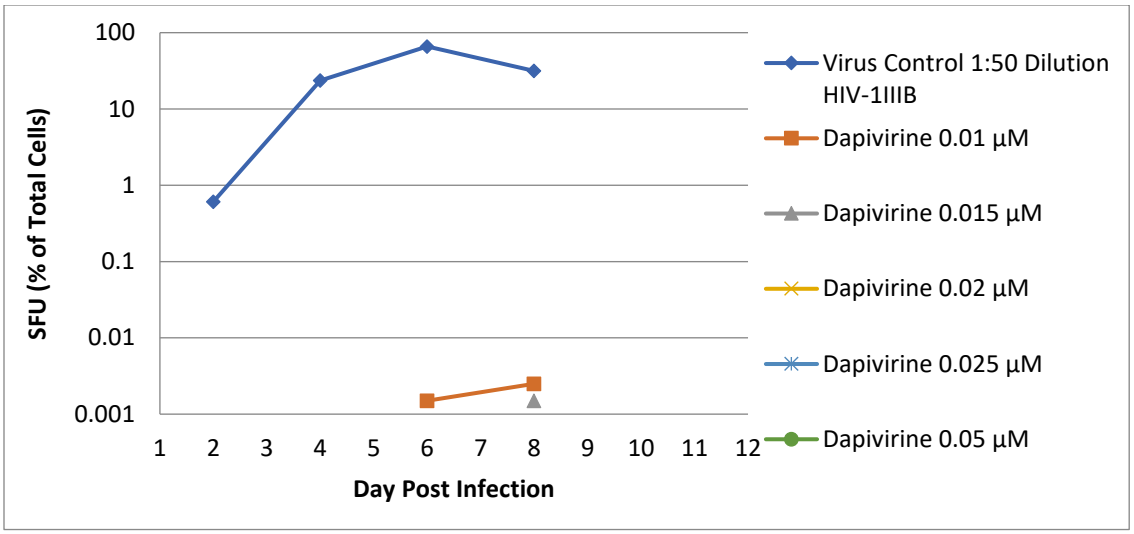


Figure 32A. Percentage of infected cells in the HIV-1_{IIIB} cultures at specified concentrations of dapivirine – Experiment two. All cultures reamined below the detectable limit for the SFU assay.

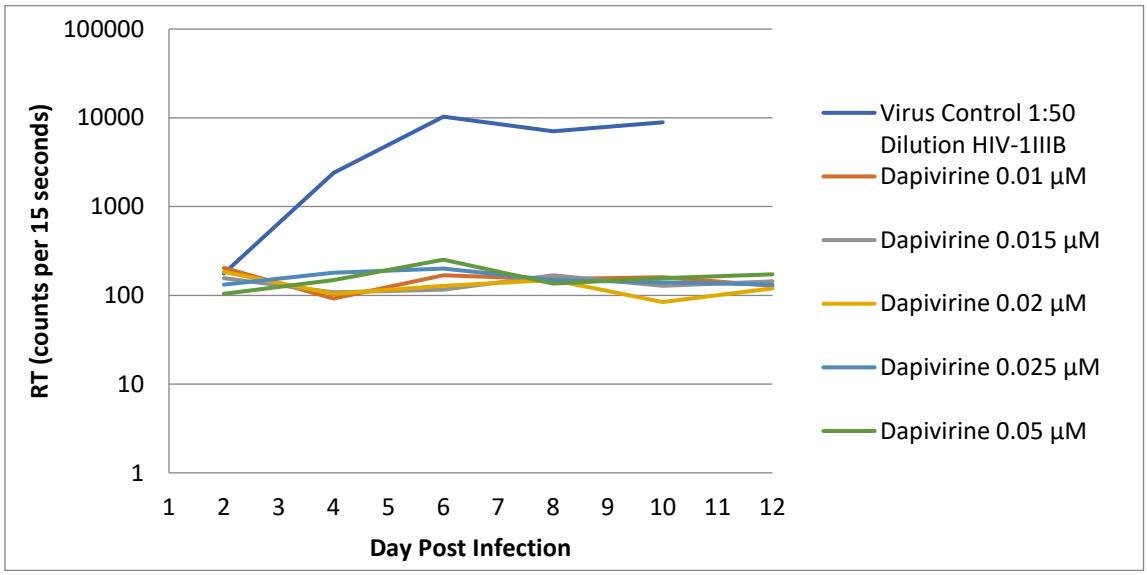


Figure 32B. RT in the HIV-1_{IIIB} cultures at specified concentrations of dapivirine– Experiment two. All cultures showed RT acivity comparable to background levels

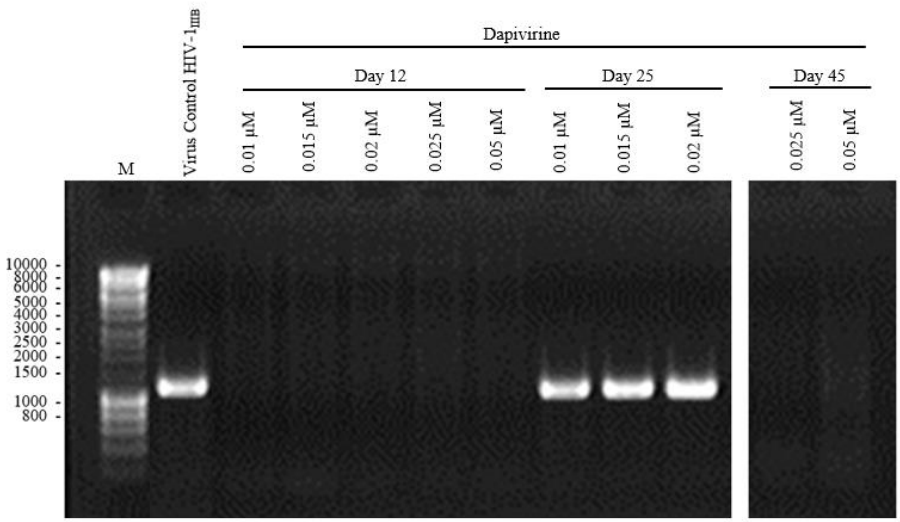


Figure 32C. PCR results of infected cells in the HIV-1_{IIIB} cultures at specified concentrations of dapivirine – Experiment two. Lane 2: Positive PCR results for the virus control on day 12 of the ICCA. Lanes 3-7: Negative PCR results for the dapivirine treated cultures on day 12 of the ICCA. Lanes 8-10: Positive PCR results for the 0.01 µM, 0.015 µM and 0.02 µM cultures on day 25 of the ICCA (day 13 post compound removal). Lanes 11- 12: Negative PCR results for the 0.025 µM and 0.05 µM treated cultures on day 45 of the ICCA (day 33 post compound removal).

HIV-1_{NL4-3}

Dapivirine was evaluated in the ICCA with wild type HIV-1_{NL4-3} in replicate experiments. The first ICCA was performed using the standard methodology with the following variations: HIV-1_{NL4-3} was used at a dilution of 1:2 to infect the CEM-SS cells and dapivirine was evaluated at 0.001 μM, 0.005 μM, 0.01 μM, 0.025 μM, 0.05 μM, and 0.1μM. The cultures were monitored for cell density and viability (not shown), syncytium formation and RT in cell free supernatants.

The culture treated with dapivirine at 0.001 μM had peak infection on day 6 of the assay with greater than 50% of the cells in the culture infected. The culture treated with dapivirine at 0.005 μM had peak infection on day 8, with greater than 0.1% of the cells in the culture infected. The culture treated with dapivirine at 0.01 μM had peak infection on day 12, slightly above the limit of detection. The remaining dapivirine treated cultures remained below the detectable limit in the SFU assay. These data are presented in Figure 33A.

Peak RT was observed in the culture treated with dapivirine at 0.001 μM on day 12. The remaining cultures at various dapivirine concentrations had RT comparable to background levels. These data are presented in Figure 33B.

PCR performed on samples collected from the cultures on day 12 confirmed all cultures to be infected by HIV. These data are presented in Figure 33C.

Infection of the treated cultures was confirmed on day 16 of the assay (day 4 post compound removal) using PCR, but no CPE were observed in the cultures. These data are presented in Figure 33C.

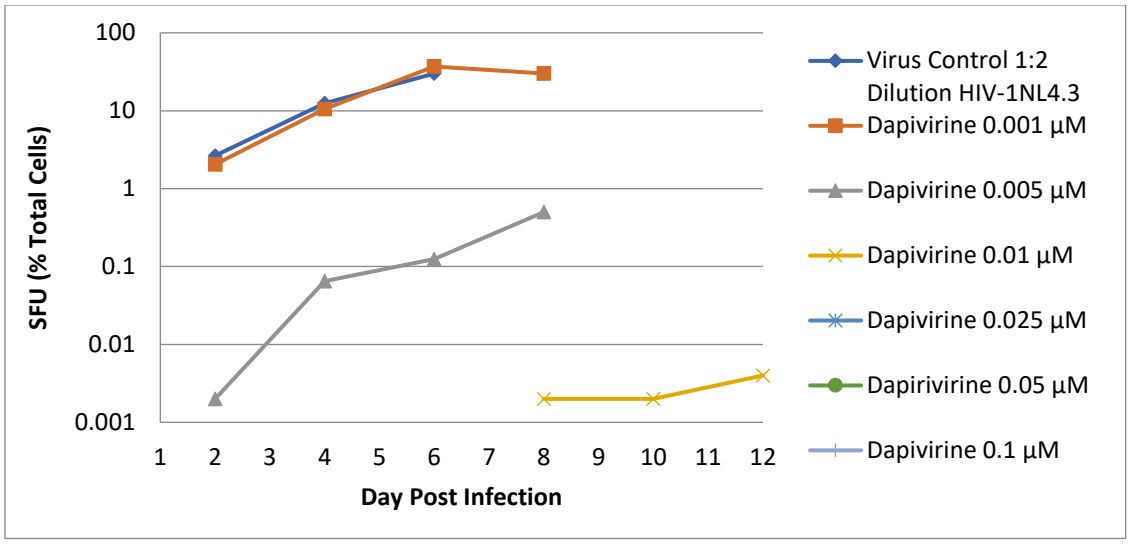


Figure 33A. Percentage of infected cells in the HIV-1_{NL4-3} cultures at specified concentrations of dapivirine – Experiment one. The 0.001 µM treated cultures had peak infection on day 6. The 0.005 µM treated culture had peak infection on day 8. All other dapivirine treated cultures remained below the detectable limit for the SFU assay.

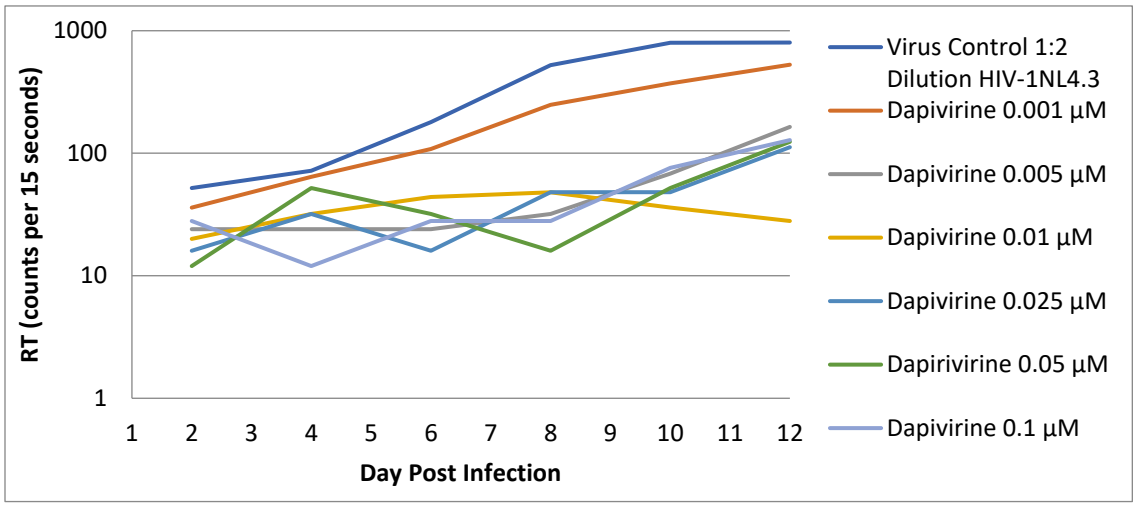


Figure 33B. RT in the HIV-1_{NL4-3} cultures at specified concentrations of dapivirine – Experiment one. Peak RT in the 0.001 µM treated culture was observed on day 12. All other cultures had RT comparable to background levels.

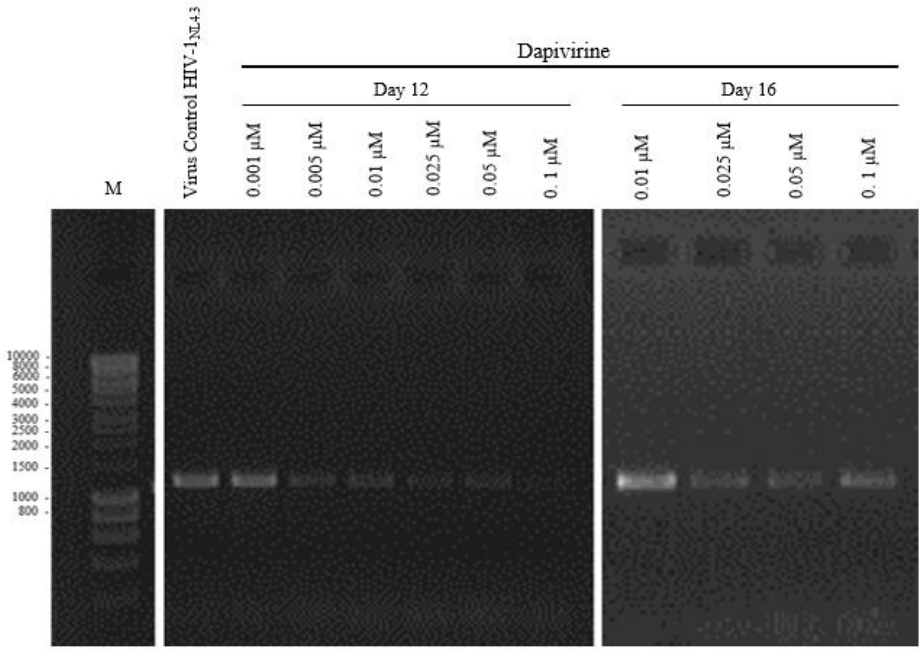


Figure 33C. PCR results of infected cells in the HIV-1_{NL4-3} cultures at specified concentrations of dapivirine – experiment one. Lane 2: Positive PCR result for the virus control culture on day 12 of the ICCA. Lanes 3-8: Positive PCR results of the dapivirine treated cultures on day 12 of the ICCA. Lane 9-12: Positive PCR product of the 0.01 µM, 0.025 µM, 0.05 µM and 0.1 µM treated culture on day 16 of the ICCA (day 4 post compound removal).

The second ICCA was performed with the same dilution of HIV-1 but the dapivirine concentrations evaluated were 0.001 μM, 0.005 μM, 0.01 μM, 0.025 μM 0.05 μM, 0.1 μM, 0.2 μM and 0.5 μM based on the results of the first experiment. The cultures were monitored for cell density and viability (not shown), syncytium formation and RT in cell free supernatants.

The culture treated with dapivirine at 0.001 μM had peak infection on day 6 of the assay with greater than 50% of the cells of the culture infected. The culture treated with dapivirine at 0.005 μM had peak infection on day 10, with greater than 1% of the cells in the culture infected. Syncytium counts above the lower limit of detection were observed in the culture treated with dapivirine at 0.01 μM from day 4 to day 12 of the ICCA. These data are presented in Figure 34A.

Peak RT was observed in the culture treated with dapivirine at 0.001 μM on day 8. All other dapivirine treated cultures had RT comparable to background levels. These data are presented in Figure 34B.

PCR evaluations confirmed that the cultures treated with dapivirine at 0.001 μM and 0.005 μM were infected with HIV on day 12. All other dapivirine treated cultures remained uninfected. These data are presented in Figure 34C.

CPE was observed in the culture treated with dapivirine at 0.01 μM on day 20 of the ICCA (8 days post compound removal). Infection of the culture treated with dapivirine at 0.01 μM was confirmed on day 20 (8 days post compound removal) by PCR. The culture treated with dapivirine at 0.025 μM was confirmed to be infected on day 29 (17 days post compound removal) by PCR. The cultures treated with dapivirine at 0.05 μM, 0.1 μM, 0.2 μM and 0.5 μM were infected based on the CPE in the cultures by day 31

(19 days post compound removal) of the ICCA and infection was confirmed by PCR. These data are shown in Figure 34C.

These results suggest that the sterilizing concentration of dapivirine is greater than 0.5 μM against HIV-1_{NL4-3}.

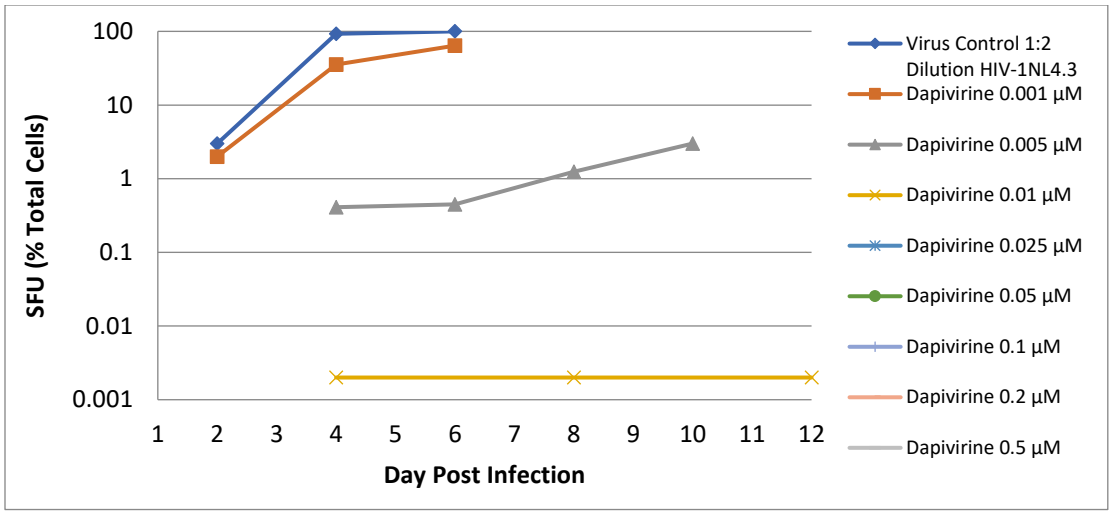


Figure 34A. Percentage of infected cells in the HIV-1_{NL4-3} cultures at specified concentrations of dapivirine – Experiment two. The 0.001 µM treated culture had peak infection on day 6. The 0.005 µM treated culture had peak infection on day 10. Cultures with dapivirine concentrations at or greater than 0.01 µM remained below the detectable limit for the SFU assay.

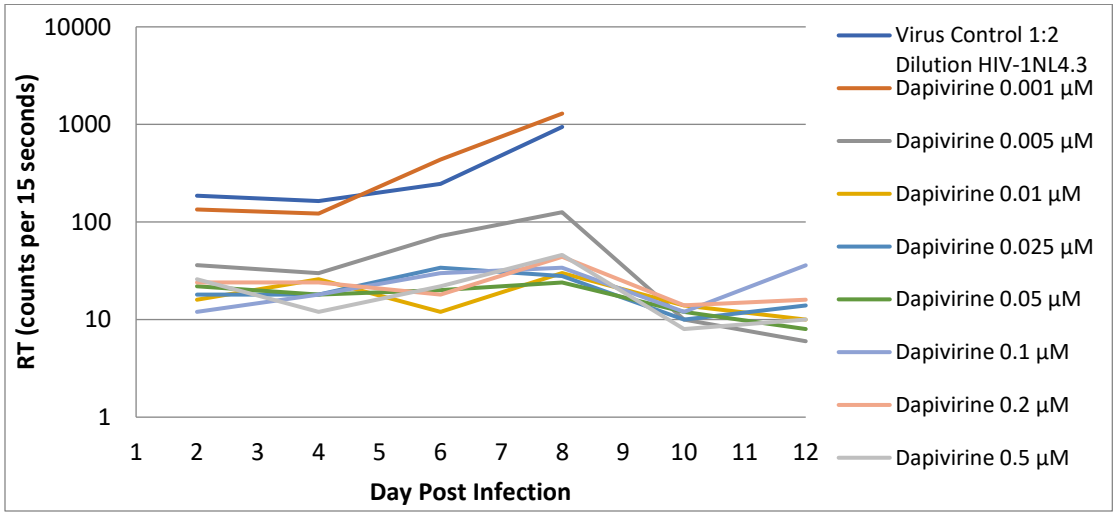


Figure 34B. RT in the HIV-1_{NL4-3} cultures at specified concentrations of dapivirine – Experiment two. The 0.001 µM treated culture had peak RT on day 8. All other cultures had RT comparable to background levels.

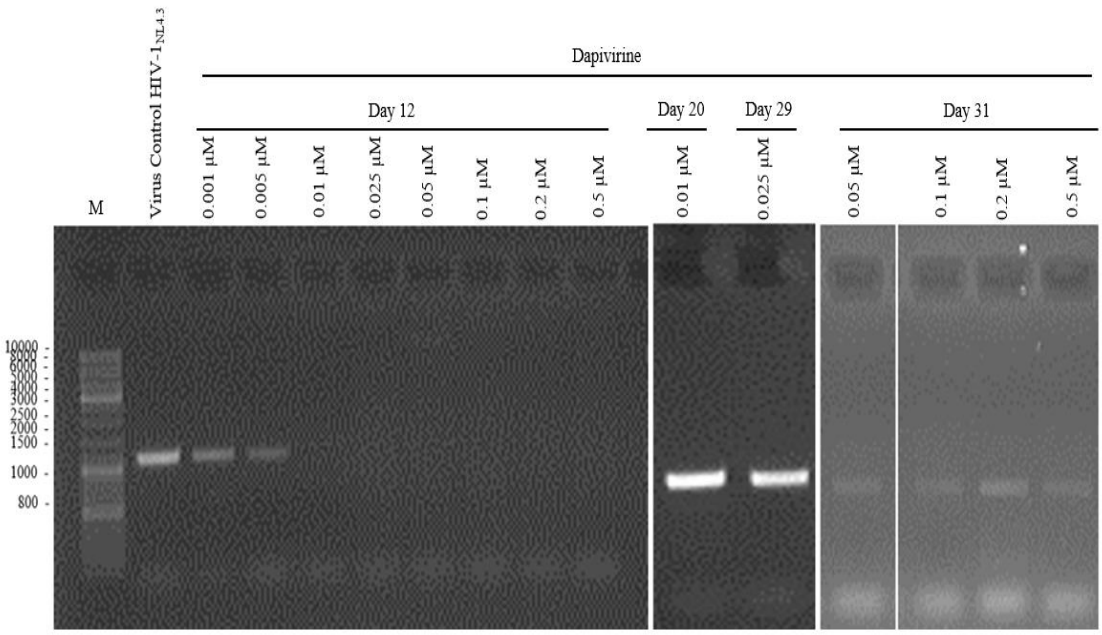


Figure 34C. PCR results of infected cells in the HIV-1_{NL4-3} cultures at specified concentrations of dapivirine – Experiment two. Lane 2: Positive PCR result for the virus control culture on day 12 of the ICCA. Lanes 3-4: Positive PCR results for the 0.001 µM and 0.005 µM dapivirine treated cultures on day 12 of the ICCA. Lane 5-10: Negative PCR results for the reamining cultures grown in the presence of dapivirine on day 12 of the ICCA. Lane 11: Positive PCR result for the 0.01 µM Dapivirine treated culture on day 20 of the ICCA (8 days post compound removal). Lane 12: Positive PCR result for the 0.025 µM treated dapivirine culture on day 29 of the ICCA (17 days post compound removal). Lane 13-16: Positive PCR results for the 0.05 µM, 0.1 µM, 0.2 µM and 0.5 µM dapivirine treated cultures on day 31 of the ICCA (19 days post compound removal).

Effects of Dapivirine in the ICCA on HIV-1 Resistant Viruses Critical to the Activity of Dapivirine

HIV-1_{Y181C}

The sterilizing concentration of dapivirine against HIV-1 with a mutation at the 181 position was evaluated in the ICCA in duplicate. The ICCA methodology was performed with the following variations: A 1:2 dilution of HIV-1_{Y181C} was used to infect the CEM-SS cells and dapivirine was evaluated at 0.005 μM, 0.01 μM, 0.05 μM, 0.1 μM, and 0.2 μM in the first ICCA and 0.01 μM, 0.05 μM, 0.1 μM, 0.2 μM, 0.5 μM and 1 μM in the second ICCA. The cultures were monitored for cell density and viability (not shown), syncytium formation and RT in cell free supernatants.

The cultures treated with dapivirine at 0.005 μM and 0.01 μM had peak infection on day 8 of the assay. The cultures treated with dapivirine at 0.05 μM culture had peak infection on day 12. All other concentrations remained below the detectable limit for the SFU assay. These data are shown in Figure 35A.

The cultures treated with dapivirine at 0.005 μM and 0.01 μM had peak RT on day 8 of the assay. All other cultures treated with dapivirine had RT comparable to background levels. These data are shown in Figure 35B.

Infection in the cultures was confirmed using PCR on samples collected on day 12. The cultures treated with dapivirine at 0.005 μM and 0.01 μM treated cultures were confirmed to be infected on day 12. All other dapivirine treated cultures were confirmed to be uninfected. These data are shown in Figure 35C.

Compound was removed from all cultures determined to be negative for infection but by day 26 (day 14 post compound removal), all cultures were determined to be infected due to the appearance of CPE in the culture.

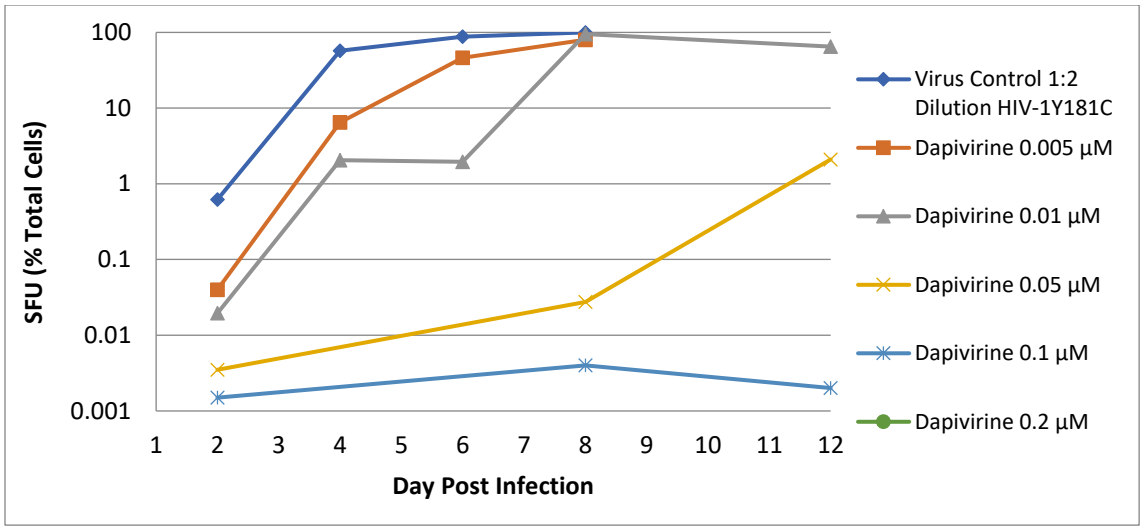


Figure 35A. Percentage of infected cells in the HIV-1_{Y181C} cultures at specified concentrations of dapivirine – Experiment one. The 0.005 µM and 0.01 µM treated cultures had peak infection on day 8. The 0.05 µM treated culture had peak infection on day 12. All other cultures remained below the detectable limit for the SFU assay.

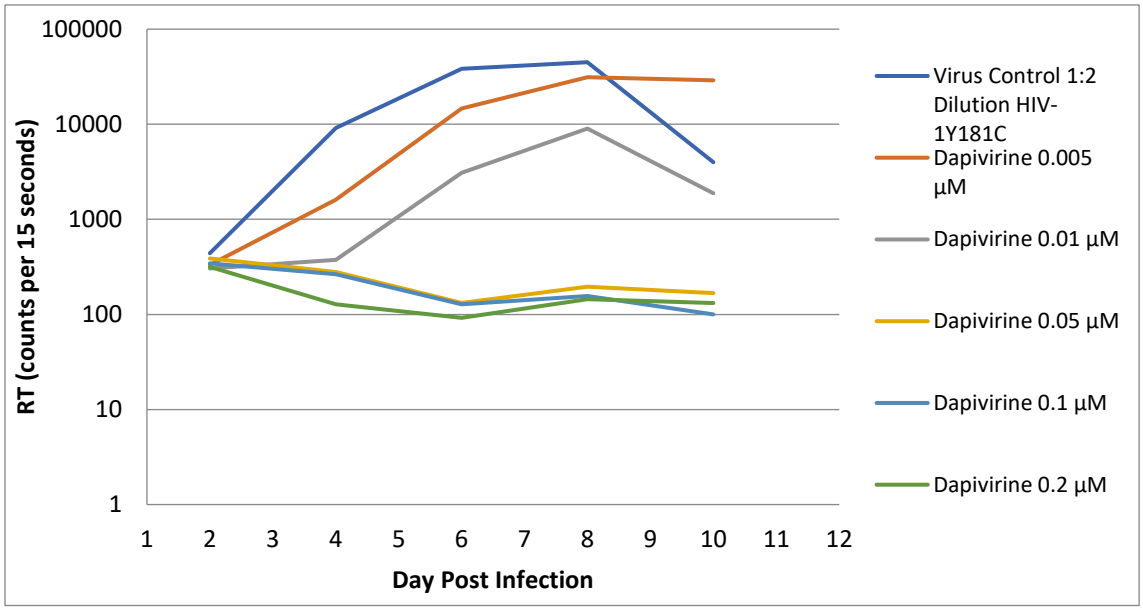


Figure 35B. RT in the HIV-1_{Y181C} cultures at specified concentrations of dapivirine – Experiment one. The 0.005 µM and 0.01 µM treated cultures had peak RT on day 8. All other dapivirine treated cultures had RT comparable to background levels.

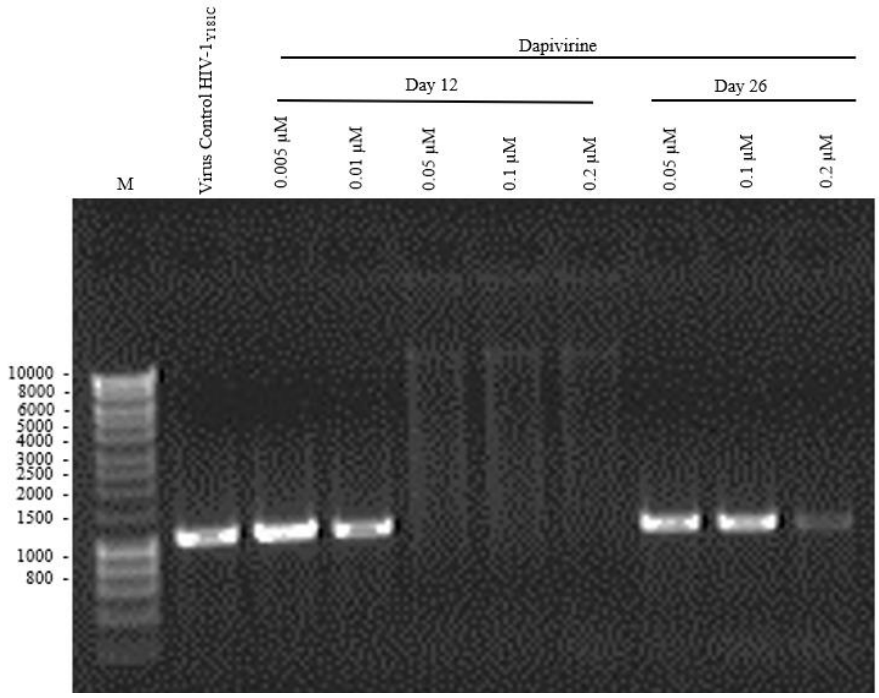


Figure 35C. PCR results of infected cells from the HIV-1_{Y181C} cultures at specified concentrations of dapivirine – Experiment one. Lane 2: Positive PCR results for the virus control culture at day 12 of the ICCA. Lanes 3- 4: Positive PCR results for the 0.005 µM and 0.01 µM dapivirine treated cultures. Lane 5-7: Negative PCR product for the remaining dapivirine treated cultures. Lanes 8- 10: Positive PCR results of the 0.05 µM, 0.01 µM and 0.2 µM dapivirine treated cultures on day 26 of the ICCA (day 14 post compound removal).

In the second experiment, the cultures treated with dapivirine at 0.01 μM, 0.05 μM, 0.1 μM and 0.2 μM had peak infection on day 12. The cultures treated with dapivirine at 0.5 μM and 1 μM remained below the limit of detection. These data are shown in Figure 36A.

The cultures treated with dapivirine at 0.01 μM and 0.05 μM had peak RT on day 8. The cultures treated with dapivirine at 0.1 μM had increased RT compared to the other cultures on day 10 and day 12, with a peak in activity on day 8. All other dapivirine treated cultures had RT comparable to background levels. These data are shown in Figure 36B.

PCR on samples taken from the treated cultures on day 12 confirmed that only the cultures treated with dapivirine at 0.01 μM was infected. These data are shown in Figure 36C.

The cultures treated with dapivirine at 0.1 μM became infected on day 16 (day 4 following compound removal) based on the presence of CPE in the culture which was confirmed by PCR. All other dapivirine treated cultures remained uninfected through 40 days. These data are shown in Figure 36C.

These results suggest that the sterilizing concentration of dapivirine in the ICCA against HIV-1_{Y181C} ranges from 0.2 μM to 0.5 μM.

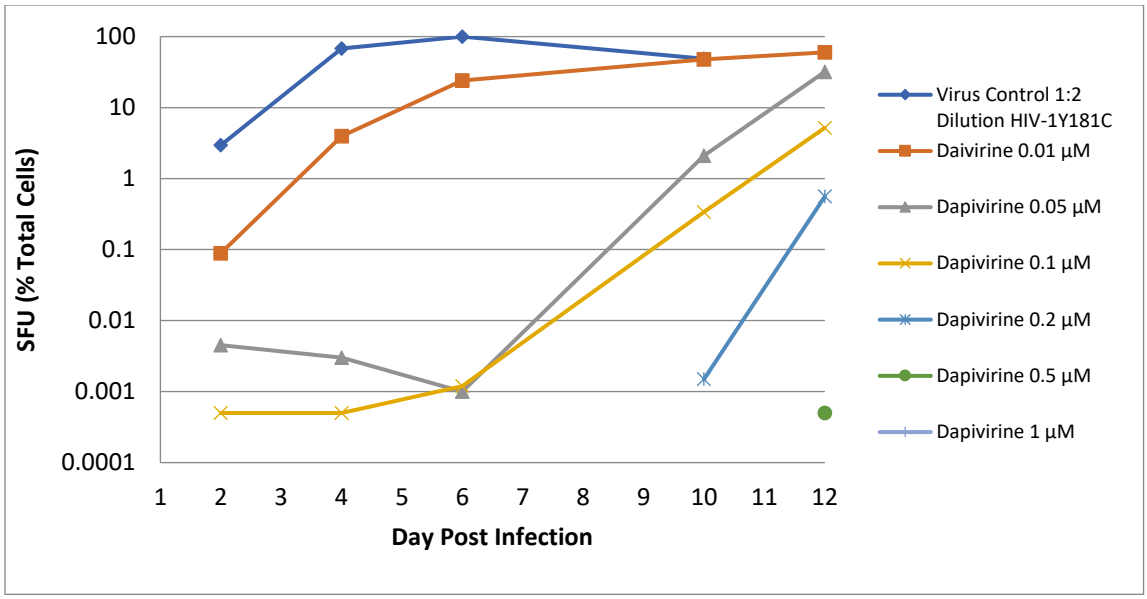


Figure 36A. Percentage of infected cells in the HIV-1_{Y181C} cultures at specified concentrations of dapivirine – Experiment two. The 0.01 µM, 0.05 µM, 0.1 µM and 0.2 µM cultures had peak infection on day 12. The 0.5 µM and 1 µM treated culture remained below the limit of detection for the SFU assay.

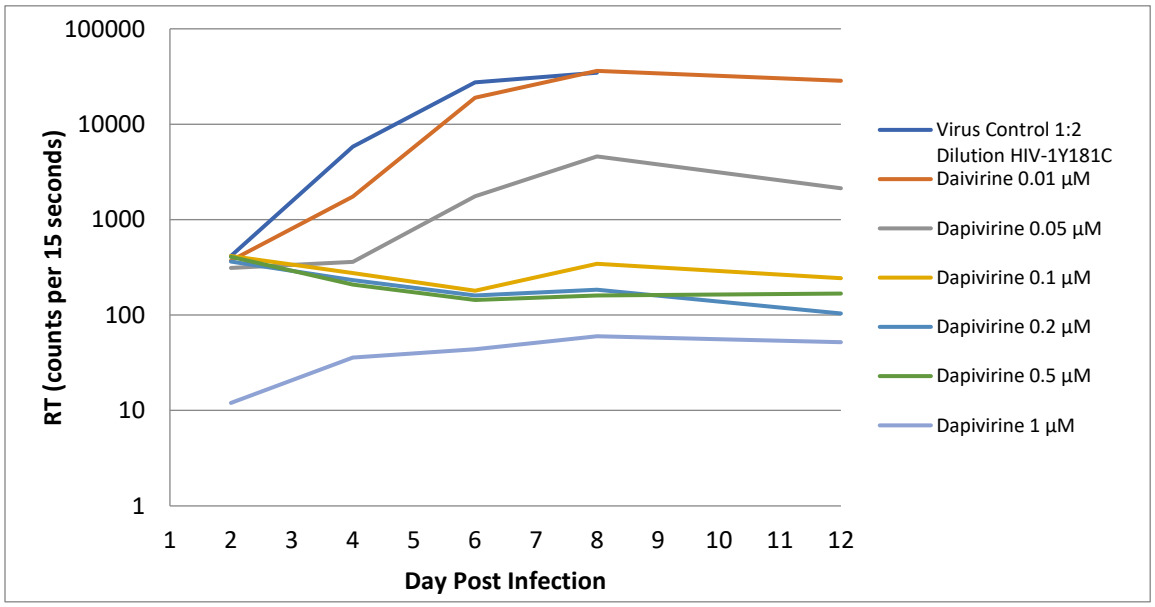


Figure 36B. RT in the HIV-1_{Y181C} cultures at specified concentrations of dapivirine – Experiment two. The 0.01 µM and 0.05 µM cultures had peak RT on day 8. The 0.1 µM treated culture had increased RT compared to the other cultures on day 10 and 12, with peak activity on day 8. All remianing cultures had RT comparable to background levels.

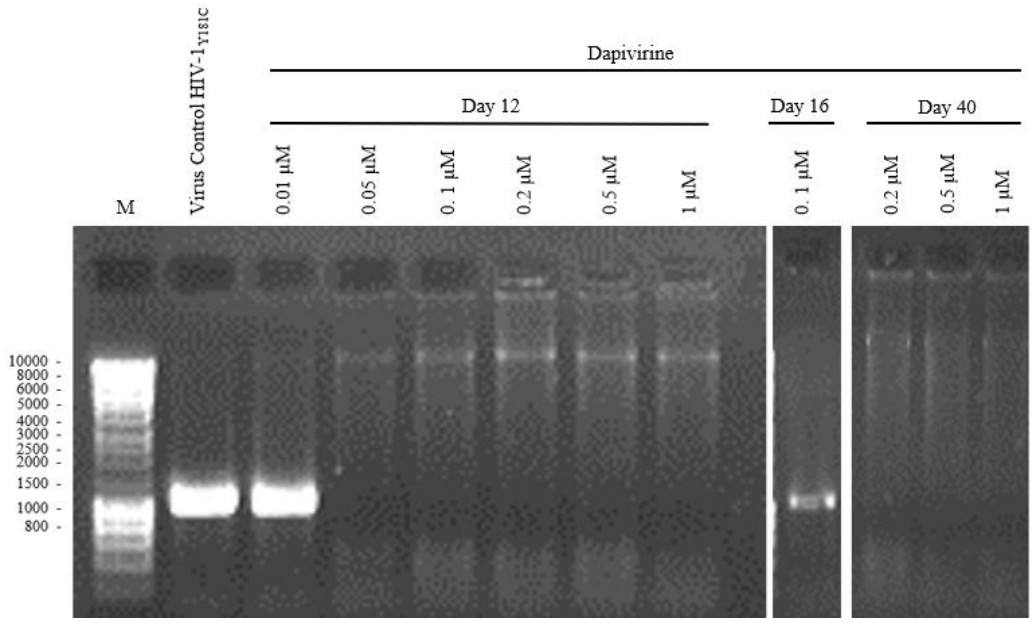


Figure 36C. PCR results of infected cells in the HIV-1_{Y181C} cultures at specified concentrations of dapivirine – Experiment two. Lane 2: Positive PCR results of the virus control culture on day 12 of the ICCA. Lanes 3: Positive PCR result of the 0.01 µM dapivirine treated culture on day 12 of the ICCA. Lane 4-8: Negative PCR results from the remaining dapivirine treated cultures on day 12 of the ICCA. Lane 9: Positive PCR result of the 0.1 µM dapivirine treated culture on day 16 of the ICCA (day 4 post compound removal). Lanes 10- 12: Negative PCR results from the 0.2 µM, 0.5 µM and 1 µM cultures on day 40 of the ICCA (day 28 post compound removal).

HIV-1_{K103N}

The sterilizing concentration of dapivirine in cultures infected with HIV-1_{K103N} was determined in the ICCA. This ICCA was performed in replicate experiments using the standard method with the following modifications: HIV-1_{K103N} at a dilution of 1:2 was used to infect the cultures, with concentrations of dapivirine including 0.005 μM, 0.01 μM, 0.05 μM, 0.1 μM, and 0.2 μM dapivirine. In the second assay the same dilution of virus was used but the concentrations for dapivirine evaluated were 0.01 μM, 0.05 μM, 0.1 μM, 0.2 μM and 0.5 μM. The cultures were monitored for cell density and viability (not shown), syncytium formation and RT in cell free supernatants.

The cultures treated with dapivirine at 0.005 μM had peak infection on day 6 with greater than 1% of the cells in the culture infected. The cultures treated with dapivirine at 0.01 μM had peak infection on day 12 with greater than 10% of the cells in the culture infected. All other dapivirine treated cultures remained below the detectable limit for the SFU assay. These data are shown in Figure 37A.

Peak RT was observed in the virus control culture on day 6. The cultures treated with dapivirine at 0.01 μM had RT above background levels on day 10 but returned to background activity on day 12. All other dapivirine treated cultures had RT comparable to background levels. These data are shown in Figure 37B.

PCR was performed on samples taken from the treated cultures on day 12 and confirmed all cultures to be uninfected. These data are shown in Figure 37C.

Compound removal was only performed on the cultures with concentrations of dapivirine greater than 0.01 μM. CPE was observed in the cultures treated with dapivirine at 0.005 μM and 0.01 μM before day 12 of the ICCA, but these cultures

became compromised by tissue culture contamination on day 8 and 12 of the assay, respectively. All other dapivirine treated cultures were washed and remained negative through visual observation of CPE in the cultures through 40 days.

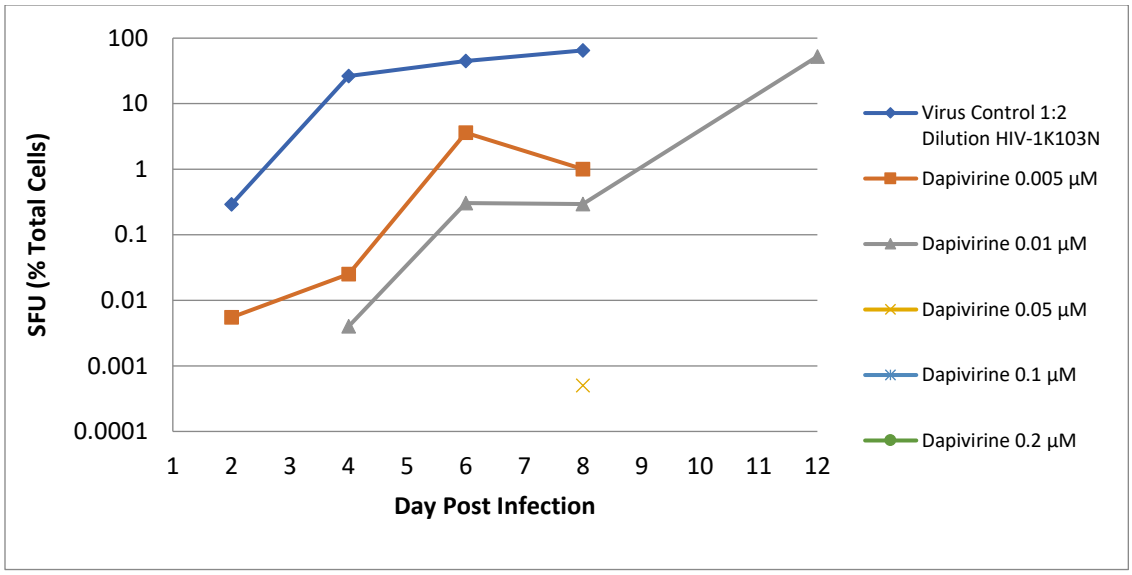


Figure 37A. Percentage of infected cells in the HIV-1_{K103N} cultures at specified concentrations of dapivirine – Experiment one. The 0.005 µM treated cultures had peak infection on day 6. The 0.01 µM treated culture had peak infection on day 12. All other dapivirine treated culture remained below the detectable limit for the SFU assay.

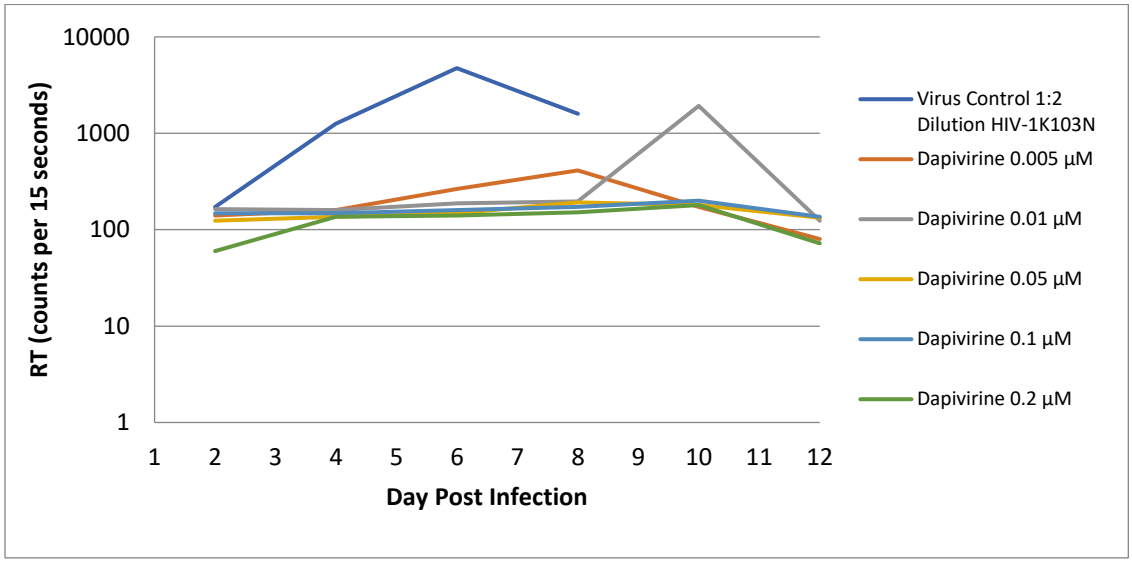


Figure 37B. RT in the HIV-1_{K103N} cultures at specified concentrations of dapivirine – Experiment one. The 0.01 µM treated culture had an increase in RT on day 10 but decreased in RT on day 12. All other dapivirine cultures had RT comparable to background levels.

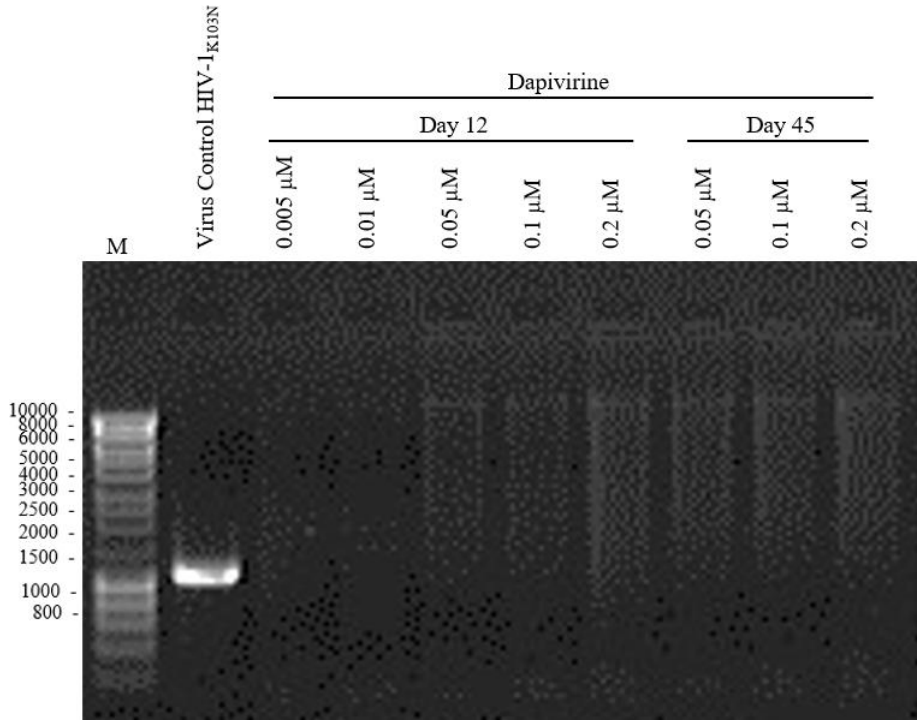


Figure 37C. PCR results of infected cells in the HIV-1_{K103N} cultures at specified concentrations of dapivirine – Experiment one. Lane 2: Positive PCR result for the virus control culture on day 12 of the ICCA. Lanes 3-7: Negative PCR results for the dapivirine treated cultures at day 12 of the ICCA. Lanes 8-10: Negative PCR results for the 0.05 µM, 0.1 µM and 0.2 µM treated cultures on day 47 of the ICCA (day 35 post compound removal).

In the second experiment, the cultures treated with dapivirine at 0.01 µM had peak infection on day 12 with greater than 1% of the cells in the culture infected. All other cultures remained below the detectable limit for the SFU assay. These data are presented in Figure 38A.

Peak RT was observed on day 6 of culture for the virus control culture. All cultures had RT comparable to background levels. These data are presented in Figure 38B.

PCR was performed on samples from the treated cultures on day 12 and confirmed all of the treated cultures to be uninfected. These data are presented in Figure 38C.

Following compound removal, the cultures treated with dapivirine at 0.01 µM was positive for CPE on day 22 (10 days post compound removal). Infection was confirmed using PCR. All other dapivirine treated cultures remained uninfected through 40 days.

These data suggest that the sterilizing concentration of dapivirine is between 0.01 µM and 0.05 µM against the drug resistant virus HIV-1_{K103N}. This is similar to the sterilizing concentration observed in the presence of HIV-1_{IIIB}.

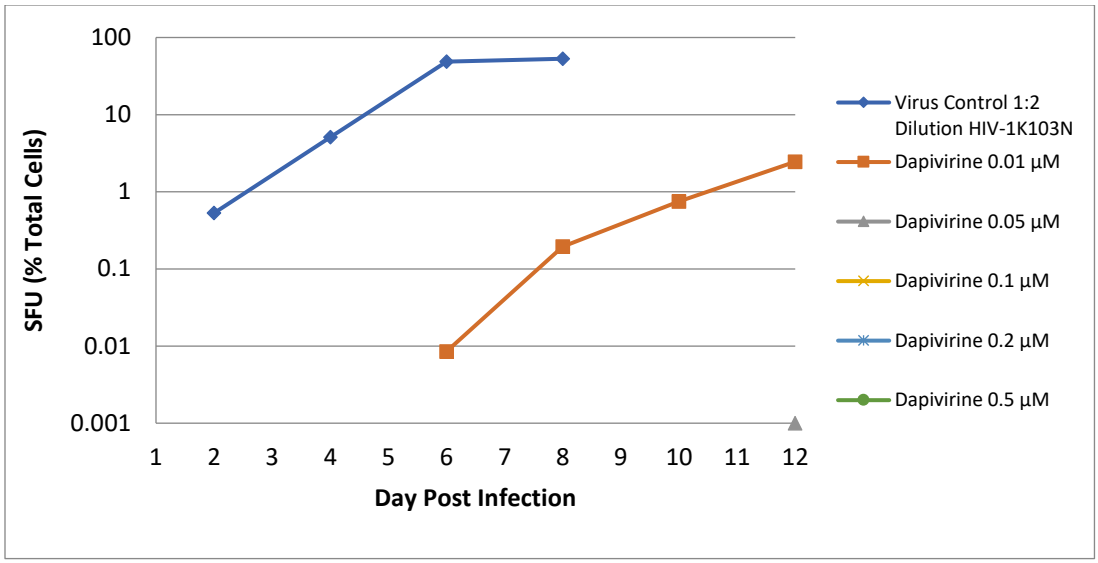


Figure 38A. Percentage of infected cells in the HIV-1_{K103N} cultures at specified concentrations of dapivirine – Experiment two. The 0.01 µM treated culture had peak infection on day 12. All other dapivirine treated cultures remained below the detectable limit for the SFU assay.

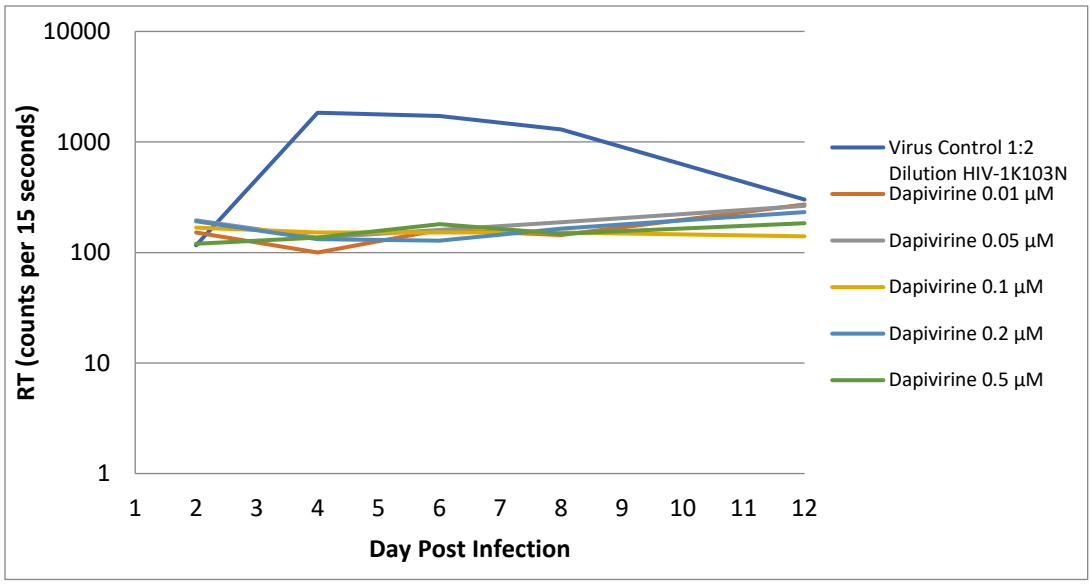


Figure 38B. RT in the HIV-1_{K103N} cultures at specified concentrations of dapivirine – Experiment two. All cultures had RT comparable to background levels.

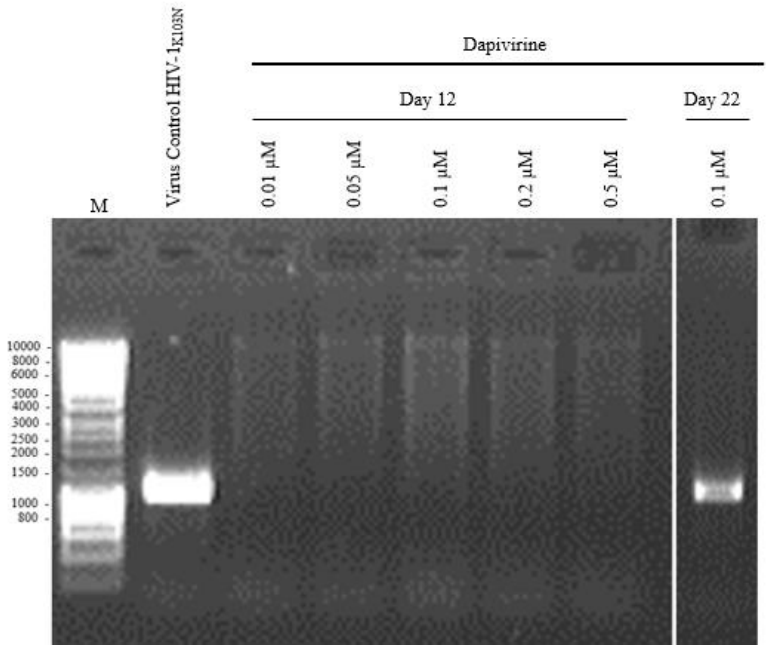


Figure 38C. PCR results of infected cells in the HIV-1_{K103N} cultures at specified concentrations of dapivirine – Experiment two. Lane 2: Positive PCR results for the virus control culture on day 12 of the ICCA. Lanes 3-7: Negative PCR results from the dapivirine treated cultures on day 12 of the ICCA. Lane 8: Positive PCR result from the 0.01 µM treated culture on day 22 of the ICCA (day 10 post compound removal).

A17

The sterilizing concentration of dapivirine in cultures infected with HIV-1_{A17} (HIV-1 with Y181C and K103N mutations) was determined in the ICCA. This ICCA was performed in duplicate using the standard methodology, with the following modifications: in the first ICCA the dilution of HIV-1_{A17} used was a 1:5 and the concentrations of dapivirine evaluated were 0.005 μM, 0.01 μM, 0.05 μM, 0.1 μM and 0.2 μM; in the second ICCA, the same dilution of virus was used and the concentrations of dapivirine evaluated were 0.2 μM, 0.5 μM, and 1 μM. These cultures were monitored for cell density and viability (not shown), syncytium formation and RT in cell free supernatants.

All dapivirine treated and infected cultures had peak infection on day 6 with infection ranging from greater than 1% to greater than 50% of the cells in the cultures infected. These data are presented in Figure 39A.

Peak RT was observed in the cultures treated with dapivirine at 0.005 μM and 0.01 μM on day 6. The cultures treated with dapivirine at 0.05 μM and 0.1 μM had peak RT on day 6, but the activity was only slightly above background level. The culture treated with dapivirine at 0.2 μM treated culture had RT comparable to background levels. These data are presented in Figure 39B.

PCR was performed on samples obtained from each culture and all were confirmed to be infected on day 12. These data are presented in Figure 39C.

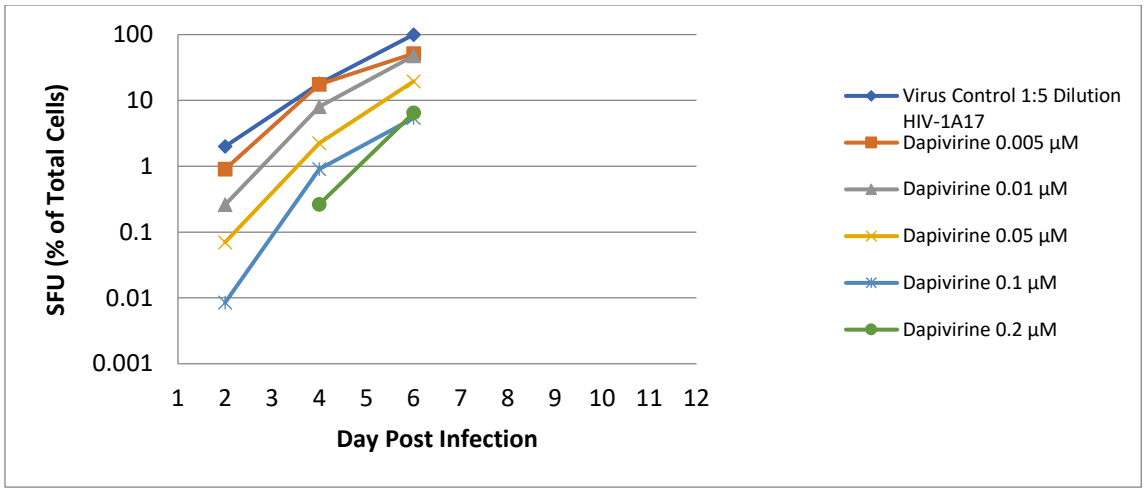


Figure 39A. Percentage of infected cells in the HIV-1_{A17} cultures at specified concentrations of dapivirine – Experiment one. All cultures had peak infection on day 6.

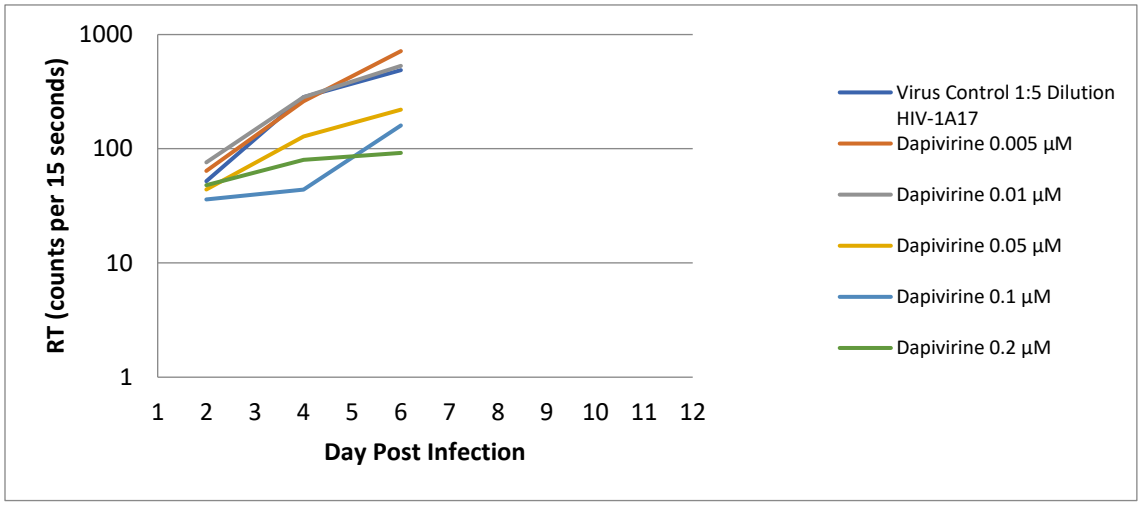


Figure 39B. RT in the HIV-1_{A17} cultures at specified concentrations of dapivirine – Experiment one. The 0.005 µM and 0.01 µM treated cultures had peak RT on day 6. The 0.05 µM and 0.1 µM treated cultures had peak RT on day 6, but this activity was only slightly above background levels. The 0.2 µM treated culture had RT comparable to background levels.

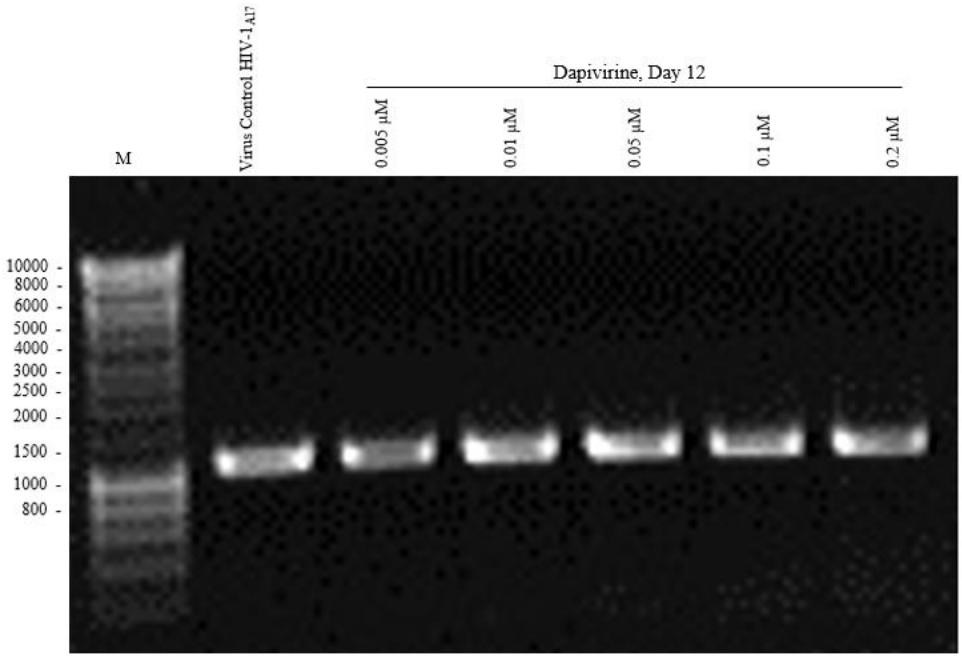


Figure 39C. PCR results of infected cells in the HIV-1_{A17} cultures at specified concentrations of dapivirine – Experiment one. Lane 2: Positive PCR result from the virus control culture on day 12 of the ICCA. Lanes 3-7: Positive PCR results for the 0.005 µM, 0.01 µM, 0.05 µM, 0.1 µM and 0.2 µM treated cultures on day 12 of the ICCA.

In the second experiment, the cultures treated with dapivirine at 0.2 µM had peak infection on day 8 with 100% of the culture infected. The cultures treated with dapivirine at 0.5 µM had peak infection on day 12 with greater than 1% of the culture infected. The cultures treated with dapivirine at 1.0 µM remained below the limit of detection for the SFU assay. These data are presented in Figure 40A.

Peak RT in the virus control culture was on day 8. The culture treated with dapivirine at 0.2 µM had peak RT on day 12. The cultures treated with dapivirine at 0.5 µM and 1 µM had RT comparable to background levels. These data are presented in Figure 40B.

PCR was performed on the samples collected from the treated cultures on day 12 and confirmed all cultures to be infected by HIV. Data are shown in Figure 40C.

Compound was removed from the 1 µM culture and on day 17 (day 5 post compound removal) CPE in the culture was observed. This result was confirmed by PCR.

These results suggest that the sterilizing concentration of dapivirine is greater than 1 µM in the ICCA in the presence of HIV-1_{A17}.

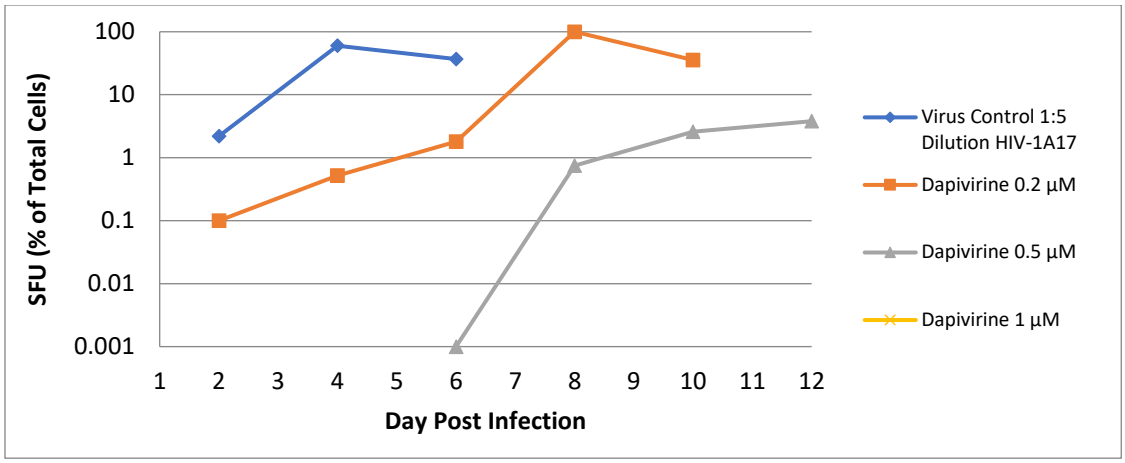


Figure 40A. Percentage of infected cells in the HIV-1_{A17} cultures at specified concentrations of dapivirine – Experiment two. Peak infection was observed on day 8 in the 0.2 µM treated culture. The 0.5 µM treated culture had peak infection on day 12. The 1 µM treated culture remained below the detectable limit for the SFU assay.

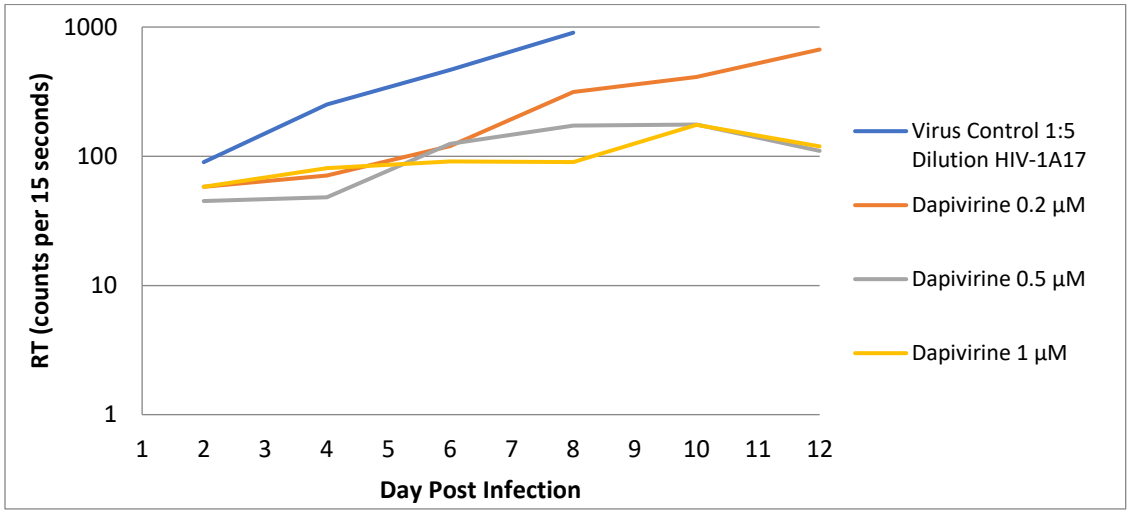


Figure 40B. RT in the HIV-1_{A17} cultures at specified concentrations of dapivirine – Experiment two. The 0.2 µM treated culture had peak RT on day 12. RT was only slightly above background levels in the 0.5 µM and 1 µM treated cultures.

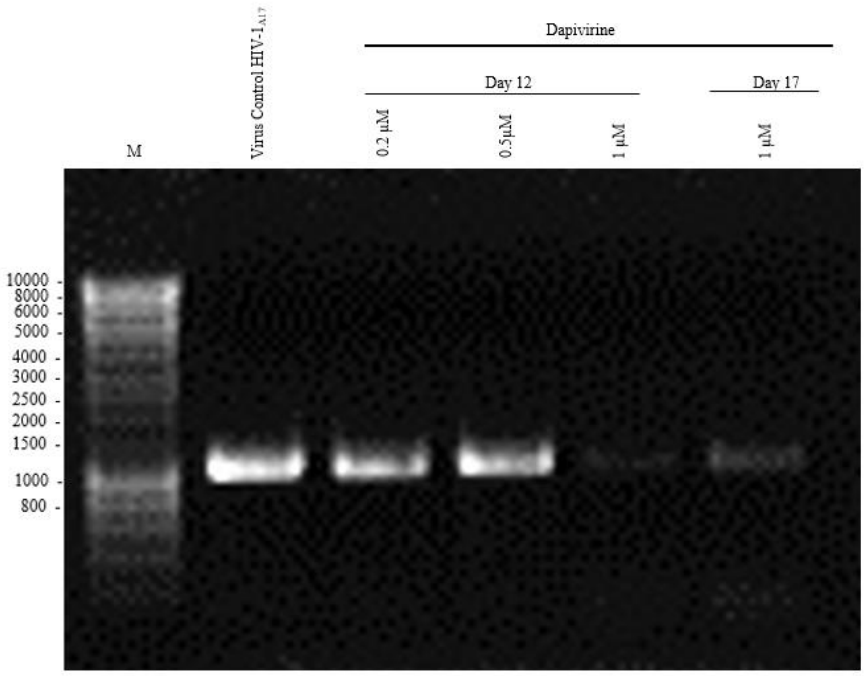


Figure 40C. PCR results of infected cells in the HIV-1_{A17} cultures at specified concentrations of dapivirine – Experiment two. Lane 2: Positive PCR result for the virus control culture on day 12 of the ICCA. Lanes 3-5: Positive PCR results for the 0.2 µM, 0.5 µM and 1 µM dapivirine treated cultures on day 12 of the ICCA. Lane 6: Positive PCR result for the 1 µM culture on day 17 of the ICCA (5 days post compound removal).

Determination of Sterilizing Concentrations of Tenofovir in the ICCA with HIV-1_{IIIB}

The sterilizing concentration of the NtRTI tenofovir was determined in the ICCA with HIV-1_{IIIB}. Two replicate assays were performed according to the standard method described with the following modifications: HIV-1_{IIIB} was used at a dilution of 1:50 to infect the CEM-SS cells and tenofovir was evaluated at 50 μM, 75 μM, 100 μM and 125 μM tenofovir. The cultures were monitored for cell density and viability (not shown), syncytium formation and RT in cell free supernatants.

The culture treated with tenofovir at 50 μM had peak infection on day 12 with 0.01% of the cells in the culture infected. These syncytium counts hovered around the limit of detection from day 2 to day 12 of the ICCA. The cultures treated with tenofovir at 75 μM, 100 μM and 120 μM remained below the detectable limit defined in the SFU assay. These data are presented in Figure 41A.

Peak RT was observed in the cultures treated with tenofovir at 50 μM and 75 μM on day 6. Peak RT was observed in the culture treated with tenofovir at 100 μM on day 10. The culture treated with tenofovir at 120 μM had peak RT on day 10, but peak RT was only slightly above background levels. These data are presented in Figure 41B.

PCR was performed on the samples from the treated cultures collected on day 12 and confirmed the cultures treated with tenofovir at 50 μM to be infected. All other tenofovir treated cultures remained uninfected. These data are presented in Figure 41C.

Following compound removal, CPE was observed in the culture treated with tenofovir at 75 μM on day 16 (4 days post compound removal) which was confirmed by PCR. The culture treated with tenofovir at 100 μM was infected by day 19 (day 7 post compound removal) as measured by CPE observed in the culture and confirmed by PCR. The cultures treated with tenofovir at 120 μM became infected on day 23 (day 11 post

compound removal) which was confirmed with both CPE in the culture and PCR. These data are presented in Figure 41C.

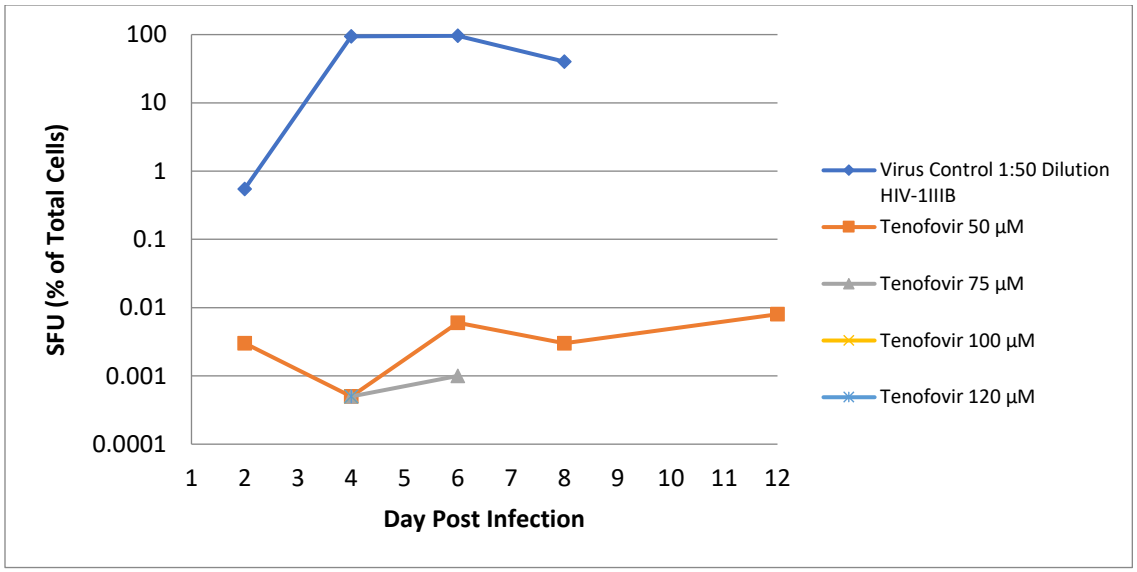


Figure 41A. Percentage of infected cells in the HIV-1_{IIIB} cultures at specified concentrations of tenofovir – Experiment one. Peak infection was observed on day 12 for the 50 µM tenofovir treated culture. All other tenofovir treated cultures remained below the limit of detection in the SFU assay.

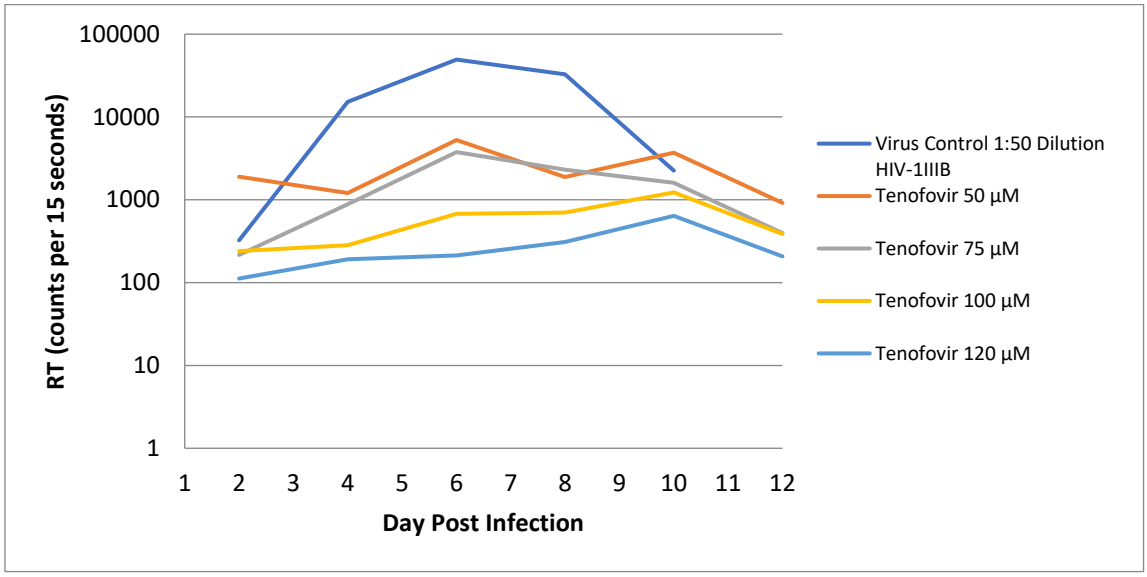


Figure 41B. RT in the HIV-1_{IIIB} cultures at specified concentrations of tenofovir – Experiment one. The 50 µM and 75 µM treated cultures had peak RT on day 6. The 100 µM and 120 µM cultures also had peak RT on day 10.

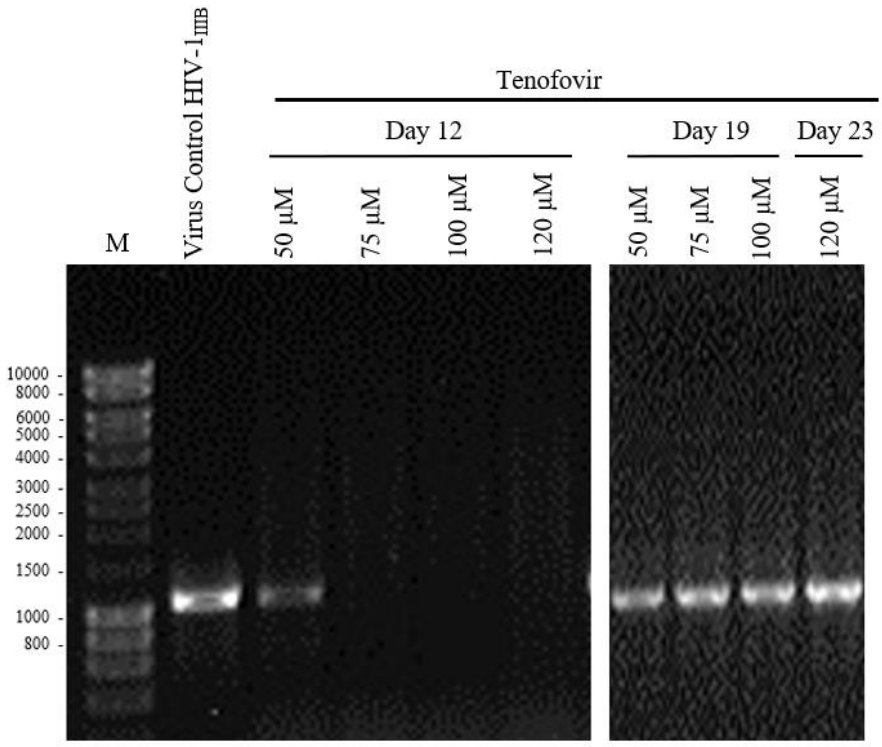


Figure 41C. PCR results of infected cells in the HIV-1_{IIIB} cultures at specified concentrations of tenofovir – Experiment one. Lane 2: Positive PCR result for the virus control culture on day 12 of the ICCA. Lane 3: Positive PCR result from the 50 µM treated culture on day 12 of the ICCA. Lane 4- 6: Negative PCR result for the remaining tenofovir treated cultures. Lanes 7-9: Positive PCR results from the 50 µM, 75 µM and 100 µM tenofovir treated cultures on day 19 of the ICCA (7 days post compound removal). Lane 10: Positive PCR results from the 120 µM culture on day 23 of the ICCA (11 days post wash).

In the second experiment, the cultures treated with tenofovir at 50 µM had peak infection on day 12 with less than 0.01% of the cells in the culture infected. The cultures treated with tenofovir at 75 µM had one count above the limit of detection on day 10. All other cultures remained below the limit of detection defined in the SFU assay. These data are presented in Figure 42A.

The cultures treated with tenofovir at 50 µM treated culture had peak RT on day 6; all other treated cultures had RT comparable to background levels. These data are presented in Figure 42B.

PCR was performed on the samples from treated cultures collected on day 12 and confirmed the culture treated with tenofovir at 50 µM to be infected. All other treated cultures remained uninfected on day 12. These data are presented in Figure 42C.

On day 15 (3 days post compound removal) CPE was observed in the cultures treated with tenofovir at 75 µM and 100 µM. Infection of these cultures was confirmed by PCR. The cultures treated with tenofovir at 120 µM became infected based on visual CPE observation of the culture on day 18 (6 days post compound removal) and this result was confirmed by PCR. These data are presented in Figure 42C.

These results suggest that the sterilizing concentration of tenofovir is greater than 120 µM in the ICCA.

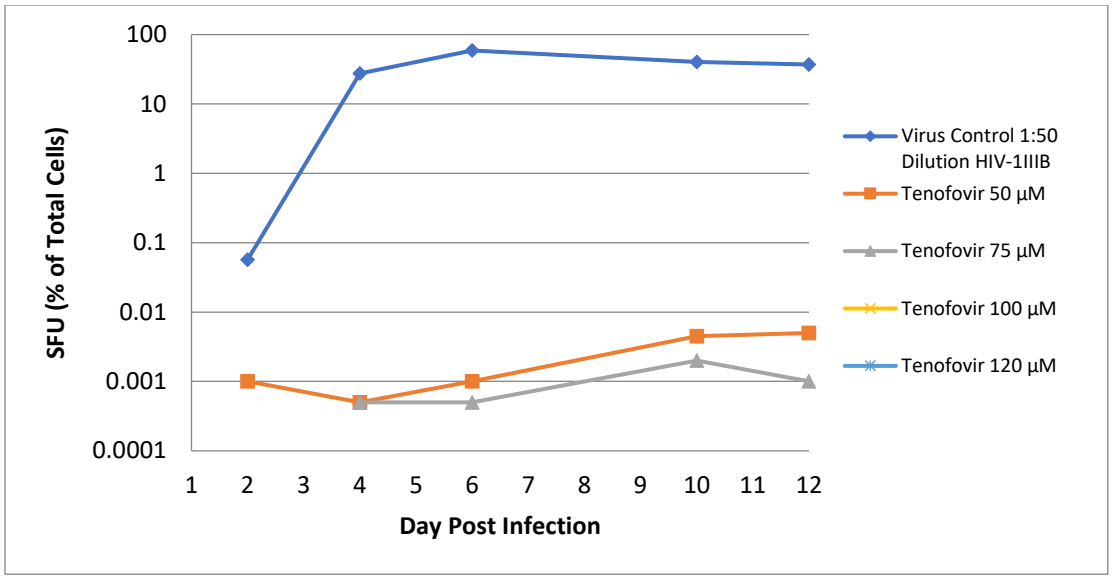


Figure 42A. Percentage of infected cells in the HIV-1_{IIIB} cultures at specified concentrations of tenofovir – Experiment two. Peak infection was observed on day 12 for the 50 µM tenofovir treated culture. The 75 µM tenofovir treated culture had one count above the limit of detection on day 10 of the ICCA. All other cultures remained below the detectable limit defined in the SFU assay.

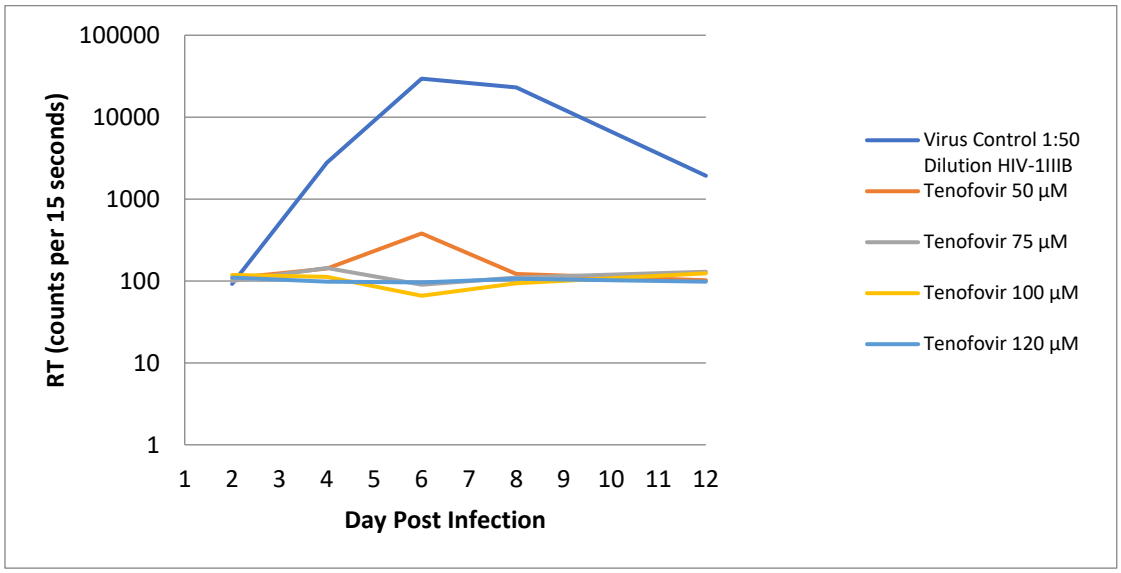


Figure 42B. RT in the HIV-1_{IIIB} cultures at specified concentrations of tenofovir – Experiment two. The 50 µM treated culture had peak RT on day 6. All other tenofovir treated cultures had RT comparable to background levels.

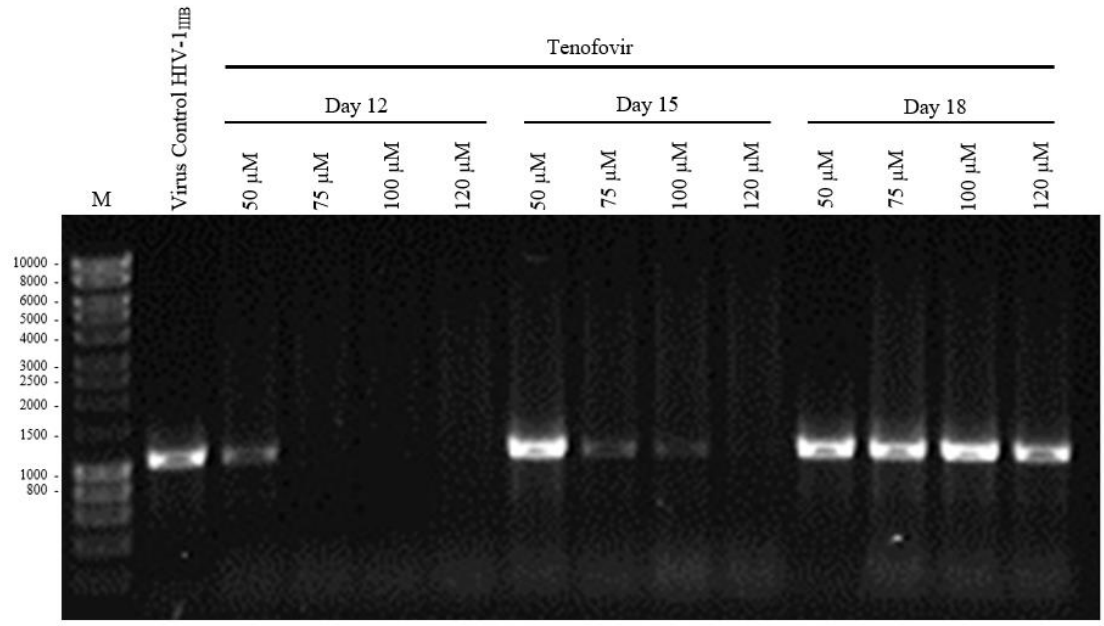


Figure 42C. PCR results of infected cells in the HIV-1_{IIIB} cultures at specified concentrations of tenofovir – Experiment two. Lane 2: Positive PCR result for the virus control culture on day 12 of the ICCA. Lane 3: Positive PCR result for the 50 µM tenofovir treated culture on day 12 of the ICCA. Lanes 4- 6: Negative PCR results for 75 µM, 100 µM and 120 µM tenofovir treated cultures. Lane 7-9: Positive PCR results from the 50 µM, 75 µM and 100 µM treated cultures on day 15 of the ICCA (3 days post compound removal). Lane 10: Negative PCR result for the 120 µM tenofovir treated culture on day 15 of the ICCA (3 days post compound removal). Lane 11-14: Positive PCR result for all tenofovir treated cultures on day 18 of the ICCA (6 days post compound removal).

Determination of the Effect of IQP-0528 and Dapivirine in Combination in the ICCA with HIV-1_{IIIB}

The effect of using a combination of the NNRTIs IQP-0528 and dapivirine was determined in the ICCA. IQP-0528 and dapivirine have identical mechanisms of action but differ in chemical structure. It was important to determine if a combination of two microbicide agents would act in an additive or synergistic fashion to sterilize a culture. This ICCA was performed according to the standard methodology described with the following variations: HIV-1_{IIIB} at a dilution of 1:50 was used to infect the CEM-SS cells and IQP-0528 and dapivirine were evaluated alone and in combination at concentrations that were known to be non-sterilizing. IQP-0528 was evaluated at 0.05 µM (2-fold below the sterilizing concentration) and dapivirine was evaluated at 0.0075 µM (3-fold below the sterilizing concentration) The cultures were monitored for cell density and viability (not shown), syncytium formation and RT in cell free supernatants.

The culture treated with IQP-0528 at 0.05 µM IQP-0528 had peak infection on day 12 with 50% of the cells of the culture infected. The culture treated with dapivirine at 0.0075 µM also had peak infection on day 12 with 20% of the cells of the culture infected. The combination of IQP-0528 and dapivirine at the same concentrations but used together resulted in a culture which was below the detectable limit defined in the SFU assay. These data are presented in Figure 43A.

Peak RT was observed in the culture treated with IQP-0528 at 0.05 µM on day 10. Peak RT was observed on day 12 in the culture treated with dapivirine at 0.0075 µM and in the combination culture utilizing of 0.05 µM of IQP-0528 and 0.0075 µM of dapivirine. These data are presented in Figure 43B.

PCR performed on samples collected from the treated cultures on day 12 of the ICCA determined the 0.05 µM IQP-0528 treated culture and the 0.0075 µM dapivirine treated culture were infected on day 12, consistent with the sub-optimal concentration of the two compounds used alone. With the combination of 0.05 µM IQP 0528 plus 0.0075 µM dapivirine the cultures remained uninfected on day 12. These data are presented in Figure 43C.

Following removal of compound, the culture with the combination of 0.05 µM IQP-0528 and 0.0075 µM dapivirine became infected as determined by observation of CPE in the culture and by PCR on day 20 (8 days post compound removal). These data are presented in Figure 43C.

These results suggest that the combination of IQP-0528 and dapivirine at concentrations below the known sterilizing concentration of each compound can delay the emergence of virus but is unable to prevent transmission at the concentrations evaluated. In light of the signal observed in the combination treated culture at day 20, it appears that a small increase in dapivirine concentration to 2-fold (rather than 3-fold) below the typical sterilizing concentration might be sterilizing.

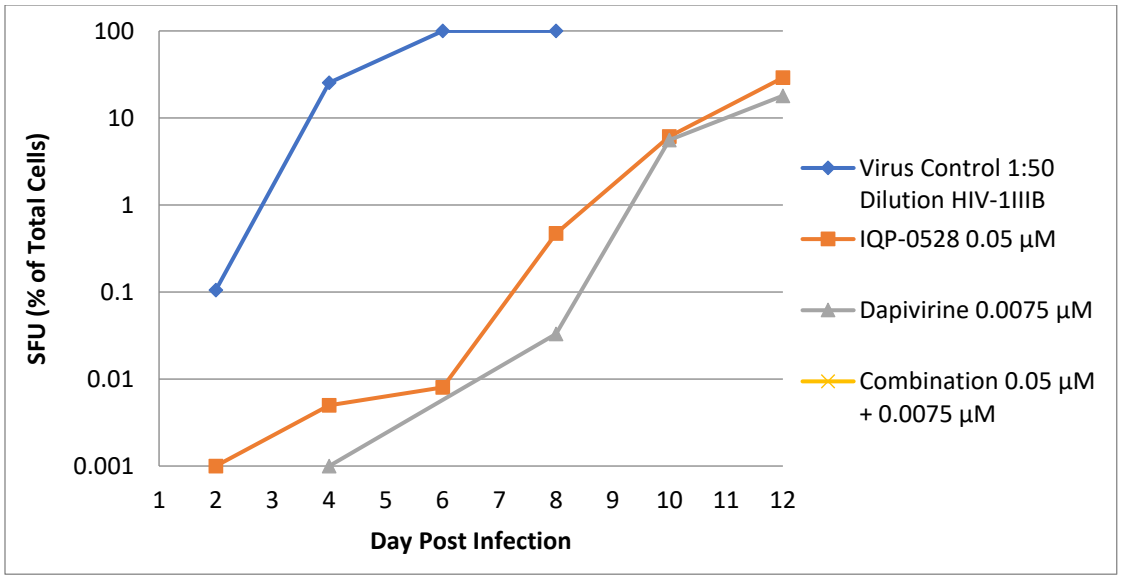


Figure 43A. Percentage of infected cells in the HIV-1_{IIIB} culture at specified concentrations of IQP-0528, dapivirine and the combination of IQP-0528 and dapivirine. The 0.05 µM IQP-0528 treated culture and the 0.0075 µM treated dapivirine culture had peak infection on day 12. The culture with the combination of 0.05 µM IQP-0528 and 0.0075 µM dapivirine remained below the limit of detection in the SFU assay.

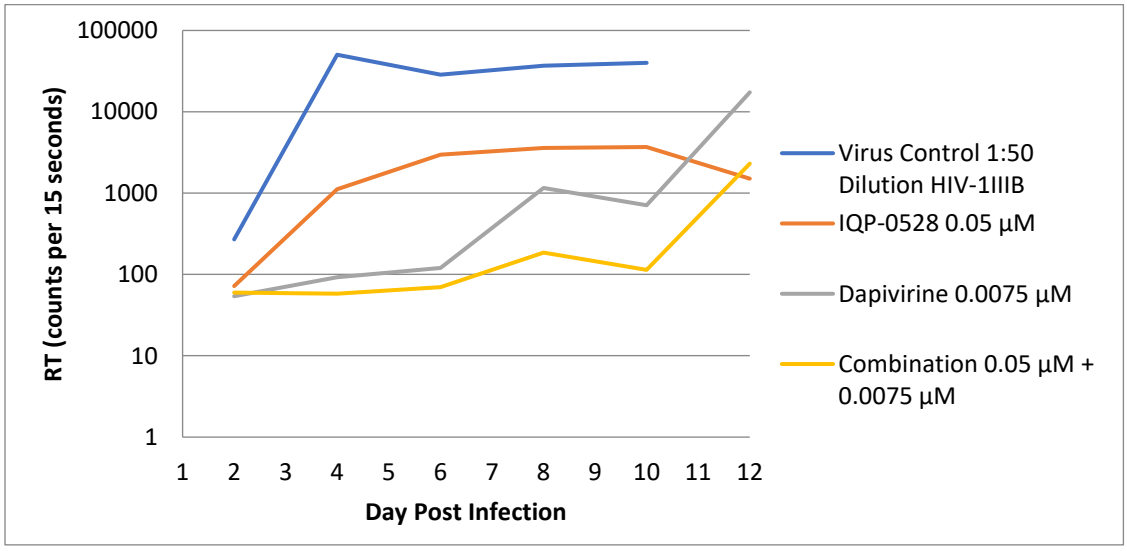


Figure 43B. RT in the infected cells in the HIV-1_{IIIB} culture at specified concentrations of IQP-0528, dapivirine and the combination of IQP-0528 and dapivirine. The 0.05 µM IQP-0528 treated culture had peak RT on day 10 and the 0.0075 µM dapivirine treated culture had peak RT on day 12. The culture with the combination of 0.05 µM IQP-0528 and 0.0075 µM dapivirine had peak RT on day 12.

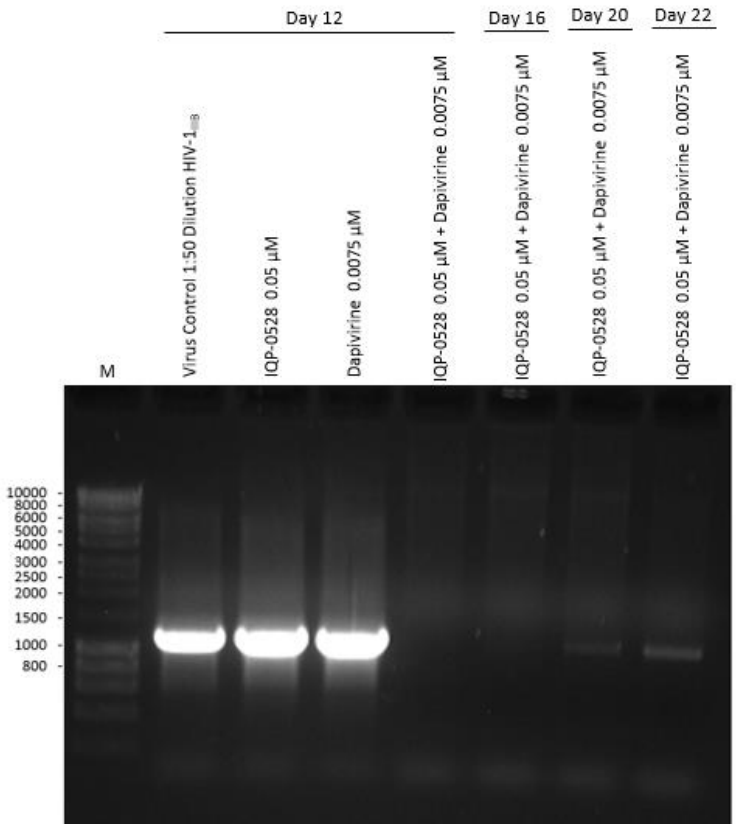


Figure 43C. PCR results of infected cells in the HIV-1_{IIIB} cultures at specified concentrations of IQP-0528, dapivirine and the combination of IQP-0528 and dapivirine. Lane 2: Positive PCR result for the virus control on day 12 of the ICCA. Lane 3: Positive PCR result of the 0.05 µM IQP-0528 treated culture on day 12 of the ICCA. Lane 4: Positive PCR result of the 0.0075 µM dapivirine treated culture on day 12 of the ICCA. Lane 5: Negative PCR result of the combination 0.05 µM IQP-0528 and 0.0075 µM dapivirine treated culture on day 12 of the ICCA. Lane 6: Negative PCR result of the combination 0.05 µM IQP-0528 and 0.0075 µM dapivirine treated culture on day 16 of the ICCA (4 days post compound removal). Lane 7: Positive PCR result of the combination 0.05 µM IQP-0528 and 0.0075 µM dapivirine treated culture on day 20 of the ICCA (8 days post compound removal). Lane 8: Positive PCR result of the combination 0.05 µM IQP-0528 and 0.0075 µM dapivirine treated culture on day 22 of the ICCA (10 days post compound removal).

DISCUSSION

With millions of infected individuals, and with increasing rates of HIV transmission around the world, the development of new and more potent HIV inhibitors is necessary. Along with development of new HIV inhibitors, novel methods of *in vitro* screening are also needed to prioritize development of these inhibitors into products. In light of the increasing rates of HIV infection in women in developing areas of the world and the lack of empowerment of those women, as well as the high transmissibility of HIV through anal sex in both women and MSM, one of the important products being developed are topically applied microbicides. Microbicides are formulated for use at the site of infection, so they must deliver a high enough concentration of inhibitor to be effective but be delivered without the chance of unnecessary and potentially dangerous systemic exposure (reviewed in Shattock and Rosenberg 2012).

A critical requirement for the use of microbicide products is that the prevention agent must be present at the site of infection at the correct time and at the correct concentration in order to prevent the transmission of HIV. Once infection occurs the virus has breached the barrier of physical or chemical protection and will freely infect underlying tissues and enter the blood stream, yielding a spreading acute systemic infection. This research project was designed and developed to determine the appropriate concentration of a microbicide drug product that is required to effectively inhibit virus transmission from occurring in the vaginal or rectal epithelial tissue. The effective concentration should totally prevent HIV from infecting target cells in the epithelial tissue and thus must be “sterilizing”. To determine the sterilizing concentration of HIV inhibitors required in a microbicide product that would totally suppress virus transmission and prevent infection in cultures, the Infectious Cell Center Assay (ICCA)

was developed, optimized and used to evaluate candidate microbicides to determine if the *in vitro* assay could be successfully utilized to determine the concentration of a microbicide needed at the tissue level to prevent transmission and infection of HIV.

Most *in vitro* antiviral assays which have been developed are best suited for the development of therapeutic anti-HIV compounds due to the fact that the assays measure the ability to inhibit initial acute infection with monitoring of virus replication and virus-induced cytopathic effects over a short period of time (from 24 hours to 6 days post infection) (Watson *et al.* 2008). Long term effects of the compound on the infected culture are not observed if the virus is able to infect target cells in the presence of the inhibitor since the assay is not designed to evaluate the eventual long-term production of virus in the infected culture. These *in vitro* assays also were not designed to have the sensitivity required to observe potential rare or low-level infection present in the cultures in the presence of the antiviral compounds. These assays report the concentrations of test and control compounds that yield 25%, 50% or 95% reduction of virus production or viral CPE in the culture (EC_{25}, EC_{50}, EC_{95}). Though these assays accurately measure the ability to suppress virus replication in a culture and clinical studies have shown the relationship between inhibition in cell culture and inhibition in human clinical trials, the assays were not designed to evaluate sterilizing inhibition which is necessary to prevent the transmission of HIV during sex (Watson *et al*. 2008). Thus, although the EC_{50} or EC_{95} concentrations are important with regards to therapeutic use of anti-HIV drugs, the relevant concentration for topical microbicide products is the EC_{100} value and many compounds cannot be utilized at a concentration that yields an EC_{100}. For example, AZT, the first approved antiretroviral, never achieves an EC_{100} concentration in cell culture and

our data shows that AZT could not be used as a topical prevention agent due to this leakiness in transmission inhibition. Thus, it is critically important to develop an assay which can define the sterilizing concentration of an anti-HIV product and then determine if that concentration can be achieved in the susceptible target tissues in *in vivo* models such as the nonhuman primate and in human clinical trials.

In considering the development of the ICCA, it was important to understand the relative multiplicity of infection (MOI) required to establish a productive infection with HIV and understand how that MOI related to the amount of virus in the human infectious inoculum (from semen). Thus, the ICCA development first required that we determine the appropriate titer of HIV to use in the assay. The initial infection of CEM-SS cells in culture was designed to be representative of the sexual transmission of HIV, with the target CEM-SS cells representing the target tissue and the infectious inoculum representing HIV present in semen as infectious virions or as infected cells. Since semen does include both free virus and virus infected lymphocytes, the ICCA was also developed to consider infection by both cell free and cell associated virus at relatively low levels of infection (0.001% infected cells in the culture) as would be expected during sexual transmission (Buckheit, Unpublished data). For most of the assays performed in this project, the HIV-1 strain used was IIIB. HIV-1_{IIIB} was chosen because it is CD4 lymphotropic, as well as syncytium inducing, and it efficiently infects CEM-SS cells (Popovic *et al.* 1984). In addition, infection with this virus in CEM-SS cells yields a cytopathic effect (syncytium formation) which results from the fusion of infected and uninfected cells and this syncytium formation allows for rapid transmission of HIV between cells in the culture. Microscopic observation of syncytium formation is a

qualitative means to determine if cell cultures are infected before quantitative testing for actual virus production (such as reverse transcriptase assay or p24 ELISA) in cell free supernatants derived from the infected culture or quantifying virus production inside the cell by polymerase chain reaction technology and visualizing amplified bands on a gel. The ICCA is essentially based on syncytium formation which assumes that a single HIV-1 infected cell will form a syncytium when mixed with an abundance of CD4-expressing uninfected cells. Like a plaque assay, the number of observed syncytia is directly proportional to the number of infected cells in the culture and thus an SFU assay may be used to quantify infected cells in a culture and the progression of infection can be measured as virus is transmitted within the culture. Our research indicated that an appropriate titer (MOI) to be used in the ICCA was a 1:50 dilution of HIV-$1_{IIIB.}$ Variations in this MOI in the ICCA resulted in changes in all biological evaluations monitored and thus variation in MOI could also be used to address potential infections with higher or lower assumed virus loads. Cultures infected at higher MOI obviously exhibited faster HIV replication kinetics and more rapid appearance of virus-induced CPE, more rapid appearance of viral proteins such as RT or p24 in cell free supernatants, and increased percentages of infected cells in the cultures with respect to time. As might be anticipated, infection at higher MOIs resulted in increased pressure on any drug to sterilize the culture and thus, the sterilizing concentration increased with increasing MOI (and conversely the sterilizing concentration decreased with reduced MOI). Our data suggest that the sterilizing concentration for any antiviral in the ICCA is MOI dependent. Importantly, the ICCA was determined to be able to distinguish between as little as a 2-fold dilution of input virus into the cultures. Differentiation between MOIs used in the

ICCA were also able to be distinguished when using a more robust strain of HIV (HIV-1_{RF}), a strain which replicates faster, to higher titers and with increased MOI when compared to HIV-1_{IIIB} infections. Our results suggest that various measurements of infection kinetics of HIV-1 are correlated: cells are initially infected at a low number (0.001-0.01%) of the total cells and the infection results in an increase in the infected cell number which increases exponentially on a daily basis. As infected cell number increases a balance exists between infected cells, total cells and syncytium formation, which results in the production of HIV and release of viral proteins into the cell free supernatant and eventual cell death and decreasing viable and total cell numbers in the culture. The critical events seem to occur when HIV infected cell number hits and surpasses the 50% to 60% infected cell number in the culture as that seems to be related to the point at which the greatest number of infected cells exist in the culture yielding highest levels of virus production and cytopathic effect. Viral MOI, effect of the presence of antiviral drugs and the relative fitness/replication capacity may all influence the time and speed at which an infected culture reaches the critical 50% infected level.

Another important variable in the development of the ICCA was defining the appropriate timing of the analysis of syncytium formation. Upon mixing a population of infected cells with a population of uninfected cells, syncytia require time to develop and become microscopically visible and quantifiable. Through our assay development and optimization evaluations, we empirically determined that the cocultivation of the populations of cells should progress for 48 hours. We determined this timepoint to be ideal and most quantitative for counting syncytia and quantifying the percentage of infected cells in the culture using the SFU assay. Allowing co-cultivation of infected

and non-infected cells for 48 hours allows for a full replication cycle of HIV in the infected cells, permitting optimal envelope glycoprotein expression and virus release from the infected cell and resulting in fusion and formation of the syncytium (Otto *et al.* 1993). At 24 hours, some infected cells may not have expressed significant amounts of envelope glycoprotein on their surface to facilitate syncytium formation or the syncytia formed may have not progressed to the point of being easily quantified, decreasing the percentage of infected cells seen. With 72 hours of co-cultivation, the results obtained were similar to those obtained at 48 hours, however, some of the syncytia would progress to cell death and degeneration. In addition, the 72 hour endpoint for the SFU might also allow for additional syncytia to be formed due to the secondary release of infectious virions into the medium from the infected cells in the SFU assay itself, thus increasing the number of syncytia above the number expected to define the percentage of infected cells from the ICCA culture. The infected cell population utilized in the ICCA was washed thoroughly before addition to the uninfected CEM-SS cells to remove excess compound and unattached, cell free virus, eliminating these two confounding variables in the assay (reduction of syncytia due to antiviral activity and/or increased syncytia due secondary infectious events from free infectious virions). Estimating the percentage of infected cells in the culture must also take into account the proliferation of the infected cell itself, yielding progeny infected cells which could amplify the number of infected cells (Watson *et al.* 2008). Since HIV forms a provirus that is integrated into the host cell genome, these daughter infected cells must be taken into account when ICCA results are tabulated and form yet another reason to restrict the assay to the 48-hour timepoint to minimize their input.

With an optimized assay with defined parameters for infection, cocultivation of the infected and uninfected cells and the timing of endpoint analysis, we evaluated the ability of representative antiretroviral agents being developed as topical microbicides to sterilize the culture and totally suppress virus replication. ImQuest BioSciences has performed significant preclinical and IND-directed studies on a pyrimidinedione NNRTI inhibitor, IQP-0528. This anti-HIV agent is characterized by a pyrimidine ring with two carbonyl groups (Buckheit *et al*. 2007a). Amongst a wide variety of analogs of the basic pyrimidinedione structure, IQP-0528 was identified as a robust and highly effective agent to be used for the prevention of HIV transmission as a topical microbicide (Buckheit *et al.* 2007a). ImQuest currently has two open INDs utilizing IQP-0528 as the API, including a vaginally applied gel and a dual vaginal/rectal applied gel. Like the entire class of NNRTIs, IQP-0528 inhibits the RT by binding noncompetitively in a hydrophobic pocket near the active site on the p66 subunit of the RT in the palm subdomain (Buckheit *et al.* 2007b). Binding of NNRTIs to this RT subunit confer conformational changes to amino acids in the pocket, specifically Y181 and Y188 residues, and also can cause changes in the thumb region of the RT resulting in a more rigid structure of the enzyme, which ultimately effects how the RT binds the RNA and effects translocation (D'Cruz and Uckun 2006). In addition to its RT inhibitory activity, laboratory results also suggest a second mechanism of anti-HIV activity of IQP-0528 which appears to involve an early occurring, pre-reverse transcription step (Buckheit *et al.* 2007b). Many assays suggest this activity may involve inhibition of virus entry of HIV through an unknown mechanism or perhaps binding of the pyrimidinedione to the RT prior to virus uncoating inside the cell, yielding early and highly efficient inhibition

of reverse transcription (Buckheit *et al.* 2007b). To various degrees, this dual mechanism of action trait is shared by all analogs within the pyrimidinedione class of inhibitors (Buckheit *et al.* 2007b). IQP-0528 also does not require a phosphorylation event to occur to confer activity, unlike the nucleoside analogs such as AZT, which requires three phosphorylation reactions upon entry to the cell and the nucleotide tenofovir which requires two phosphorylation steps (Sluis-Cremer and Tachedjian 2008). By not requiring any metabolic modifications and by being freely diffusible into target cells, IQP-0528 rapidly enters the cells and is present at the site of action before reverse transcription begins in the cytoplasm. If the early mechanism of action is correct, IQP-0528 may penetrate the viral membrane and bind the RT as it enters the cytoplasm and thus is prepared to inhibit reverse transcription immediately. Since data exist that suggest that reverse transcription actually begins prior to complete virus uncoating, should IQP-0528 penetrate into the virion and bind to the RT before cell entry occurs, its potency would be even greater since the inhibitor would be present at the same exact time as reverse transcription begins (Rankovic *et al.* 2017).

Through the MTSA, it was determined that HIV-1 transmission can be completely suppressed, and sterilization is possible in a CEM-SS cell culture treated with the NNRTI IQP-0528 (Watson *et al.* 2008). These results provided a rationale for the initial dosing experiments of IQP-0528 in the ICCA. Prevention of transmission and culture sterilization with IQP-0528 was also shown to be possible using the ICCA. Through a wide variety of ICCAs performed in the presence of IQP-0528, it was determined that the sterilizing concentration of IQP-0528 remained consistent at 0.1 µM against HIV-1_{IIIB} in the standard ICCA and was 0.05 µM against HIV-1_{NL4-3}. As noted, the sterilizing

concentration is defined as the concentration at which an inhibitor can completely inhibit virus replication and cause the immediate or eventual elimination of virus from a cell culture (Balzarini *et al*. 2003). Through optimization of these assays, it was determined that to prove sterilization of the cultures, the antiretroviral inhibitor must be removed in order to allow the resumption of virus production from any rare infected cells within the treated and infected cell population, allowing the rare infected cells to produce progeny virus and resume a productive infection. As seen in our results, cultures treated with IQP-0528 at 0.05 μM and 0.1 μM both appeared to be sterilizing on day 12 of the ICCA, but upon removal of compound through thorough washing of the cells, it was conclusively shown that the 0.05 μM culture had a rare subpopulation of infected cells which persisted in the presence of the 0.05 μM IQP-0528. Upon removal of the compound, virus infection resumed yielding an increase in virus production and a spreading infection as observed in RT activity assays and the appearance of virus-induced cytopathic effects beginning as soon as IQP-0528 was removed. In the case of the 0.1 μM treated culture, all means to detect an infected cell were found to be negative and upon removal of the compound, no evidence of a spreading infection was observed.

In addition to IQP-0528, the NNRTI dapivirine was also tested in the ICCA against HIV-1_{IIIB} and yielded a sterilization concentration of approximately 0.025 μM which was 4 to 5-fold lower than what was seen using IQP-0528. Against HIV-1_{NL4-3}, dapivirine showed a sterilizing concentration greater than 0.5 μM, 10-fold higher than what was observed with IQP-0528. With the evaluation of a second NNRTI, we showed the reproducible nature of the ICCA with multiple compounds and critically showed that replication of the sterilizing concentration was robust from assay to assay (less than a 2-

fold difference in sterilizing concentrations between different experiments). As NNRTIs, dapivirine and IQP-0528 work in identical ways but possess different molecular structures. For example, dapivirine is a diarylpyrimidine derivative, which resembles a pyrimidine nucleotide, that has been found to show potency in inhibition of HIV RT (Adams and Kashuba 2012). Dapivirine can tightly bind to the RT enzyme via an allosteric mechanism to prevent reverse transcription. This tight binding activity of dapivirine, which is likely shared by IQP-0528, was one of the antiviral features that resulted in the molecule being developed as a microbicide (D'Cruz *et al.* 2006). Also, like IQP-0528, dapivirine is highly lipophilic and insoluble in aqueous solution which renders it best used in a topical environment where uptake into the blood is not necessary (and not wanted due to the possibility of resistant virus selection) (Adams and Kashuba 2012). Direct comparison of dapivirine with IQP-0528 in our hands suggests that the two agents are equivalent from an efficacy perspective but IQP-0528 is less toxic *in vitro*, yielding a higher therapeutic index and possible clinical advantage.

In addition to the testing of NNRTIs, a nucleotide reverse transcriptase inhibitor was also used to determine if the ICCA would be amenable to defining sterilizing concentrations of compounds from different classes of HIV inhibitors. The FDA approved and widely used antiretroviral tenofovir (TFV) was chosen as a representative of the NtRTI class of HIV inhibitors, as well as in light of its utilization as a prevention and PrEP product. Tenofovir is a purine adenosine analogue and requires cell entry and intracellular metabolism (phosphorylation) to become a functional inhibitor of HIV reverse transcriptase (De Clercq, 2007). NtRTIs require one less phosphorylation event to become active compared with NRTIs. When the NtRTI is incorporated into the

growing proviral dsDNA strand being synthesized by the RT, it acts as a chain terminator because it lacks a hydroxyl group at the 3' end of the molecule preventing the continued polymerization of the growing viral DNA strand by preventing the addition of more nucleotides (Arts and Hazuda 2012). Tenofovir has a long intracellular half-life due to its phosphorylation (which traps the molecule in the cell) and can suppress viral replication without exhibiting cellular toxicity (De Clercq, 2007). These characteristics made tenofovir an ideal choice for development as a microbicide product. The clinical trial, CAPRISA 004, was completed in 2010 and determined the effectiveness and safety of a 1% tenofovir gel in uninfected, sexually active women in South Africa (Abdool *et al.* 2010). The introduction of the 1% tenofovir gel was shown to reduce HIV infection in subjects by an estimated 39% overall, and women with high adherence to the dosing regimen showed a 54% decrease with no significant adverse side effects (Abdool *et al.* 2010). There were also no changes in viral load and no resistance development observed in participants in the clinical study (Abdool *et al.* 2010). In the standard ICCA, treatment with tenofovir did not result in a sterilizing concentration up to 120 µM against HIV-1_{IIIB}. In the clinical trial performed in 2010, the 1% tenofovir gel contained 40 mg of tenofovir per dose in a hydrocellulose (HEC) gel, which is a 100-fold increase in concentration to what was evaluated in the ICCA (Abdool *et al.* 2010). This dose of TFV, was given in a coitus-dependent manner with one dose used before and one dose after sex, in order to preload epithelial tissue with drug and maintain drug in the tissue during the period HIV was introduced to target cells via semen (Abdool *et al.* 2010). Cervicovaginal fluid from women who were treated with the 1% TFV gel and had concentrations of TFV greater than 1,000 ng/mL were associated with increases in

efficacy (Kashuba *et al.* 2015). The concentration of tenofovir in the cervicovaginal fluid is a greater than 2-fold increase in concentration to what was evaluated in the ICCA. With the high levels of dosing of TFV, evaluation of sterilization in our assays may be problematic due to the toxicity of TFV to CEM-SS cells and thus, sterilization might be achieved but at the expense of nonspecific cellular toxicity. This experiment highlighted one possible limitation of the ICCA in that toxicity of compounds, and the solvents in which they are solubilized in, might limit the concentrations which can be evaluated and thus, may lead to results suggesting compounds at nontoxic concentrations do not yield sterilization. That said, toxicity observed toward cells *in vitro* may be similar to toxicity observed to cells and tissues in animals and humans. The results of our testing suggested that NtRTIs may not be able to sterilize cultures as efficiently as NNRTIs. One possible mechanism which might explain this result is the rapid induction of antiviral activity by the freely diffusible NNRTIs which suppresses virus replication from its earliest stages versus the requirement for metabolism of the NtRTI which allows the virus to get a head start and establish a reservoir of infected cells which persists and prevents complete sterilization. Once virus replication has progressed beyond reverse transcription, tenofovir would have no impact on the virus and thus an infected cell possessing an integrated provirus would be made and begin to proliferate itself.

Further evidence of the potential inferiority of nucleoside analogs as microbicide products has been observed in MTSA evaluations of AZT performed in our laboratory. AZT, a commonly prescribed NRTI and the first drug approved for use in HIV infected patients, was not used in the ICCA experiments presented herein since we had previously shown its inferiority in the MTSA. AZT is a pyrimidine nucleoside analogue of

thymidine with a higher affinity for the binding site of the RT than a natural thymidine nucleoside while showing decreased affinity for the cellular DNA polymerase, which limits disruption to the host cell processes (Bourdais *et al*. 1996). Previously published results from our laboratory suggest that the nucleoside is very ineffective in virus sterilization assays. MTSAs performed by Watson *et al*. in 2008 showed virus outgrowth in cultures containing AZT concentrations greater than 1,000 times the EC_{50} in as little as two passages (6 days) (Watson *et al.* 2008). Conclusions can be drawn that AZT would act similarly in the ICCA based on similar results in the ICCA and MTSA with other compounds. Again, the requirement for three steps of phosphorylation inside the cell presumably allows the virus to gain a significant foothold in the target cell population and resist sterilization. In this case, AZT would act similarly to tenofovir in the standard ICCA against HIB-1_{IIIB}. From the data generated as a result of these experiments it can be hypothesized that some classes of inhibitors work better than others in the standard ICCA with HIV-1_{IIIB}. The NNRTIs tested are shown to have lower sterilizing concentrations than that of the NtRTI tenofovir and what was shown historically in the MTSA for the NRTI AZT.

In addition to specific limitations of each compound, in general, studies have shown that NRTIs are not as efficacious alone as NNRTIs and work better for treatment in combination with another compound of a different class (Garg *et al.* 2009). In contrast to NRTIs, NNRTIs are active without phosphorylation events and are freely permeable in and out of the cell membrane. This allows NNRTIs to work faster and more efficiently to suppress and inhibit transmission of cell free and cell associated virus. For this reason, NNRTIs work better in the ICCA at preventing initial infection of HIV than NRTIs.

During the course of development of the ICCA we also evaluated the possibility that combinations of antiretroviral products might be more effective at sterilizing cultures than single agents. Since IQP-0528 and dapivirine were the compounds predominantly used in the studies reported herein, we evaluated the effects of this combination of agents in the ICCA. IQP-0528 and dapivirine are from the same class of agents (NNRTIs) but do appear to have slightly different mechanism of action with IQP-0528 reportedly possessing an early acting inhibitory mechanism prior to reverse transcription (Buckheit *et al.* 2007b). Dapivirine is also being developed as a microbicide ring and we wanted to determine if adding IQP-0528 could benefit this formulation. The results of the combination ICCA performed suggests that the combination of IQP-0528 plus dapivirine is highly active in suppressing HIV replication but that higher concentrations of the two products should be tested to provide sterilization. We have also evaluated the combination of FTC and TDF (currently marketed as Truvada) in the ICCA, and it was determined that alone neither FTC nor TDF could sterilize a CEM-SS cell culture at the concentrations evaluated. In combination, even the lowest concentrations of both NRTIs provided sterilization of the cultures yielding synergistic activity (Wilkinson, Unpublished Data). Additional combinations of therapeutic agents evaluated in the ICCA would provide additional evidence for combination product microbicide development, especially considering the success of HAART which involves combinations of agents. The current strategy for HAART is a combination of two NRTIs/ NtRTIs with an NNRTI for first line treatment for HIV (Bhatti *et al.* 2016). In the event of failure of the first line treatment, second line HAART treatment is used and combines two NRTIs and a protease inhibitor combined with a booster (Bhattti *et al.* 2016).

Salvage therapy is available for individuals who fail first and second line treatments, and it is recommended that this therapy include ARVs with the least risk for developing cross resistance, such as second generation NNRTIs and protease or integrase inhibitors (Bhatti *et al.* 2016)

It should be noted that the emergence of resistant virus in infected patients is a byproduct of virus replication. It is well known that the definition of an antiviral agent is a drug which results in the selection of resistant virus. In the case of prevention, the resistance issue is slightly different. Prevention is about the formation of a chemical barrier to prevent the passage of virus from semen into target cells and the initiation of a productive infection. Thus, resistance in a prevention setting is the ability of a resistant virion in the virus inoculum to preexist and thus bypass the chemical barrier by virtue of the existing mutations present in the population as opposed to a replicating virus obtaining resistance mutations. It is well known that early treatment of patients with monotherapy regimens seeded the diverse quasi-species of circulating HIV in the population with significant numbers of mutations to all classes of HIV inhibitors. Thus, appropriate combinations of prevention agents must be identified to mitigate against the risk that a virus with a high prevalence mutation that allows all compounds in the combination strategy to be inactive.

For topical microbicides, at first glance it would seem important that the active ingredients of a microbicide act prior to integration of proviral DNA into the host cell genome (Garg *et al.* 2009). This would limit the possible active ingredients to classes of inhibitors which inhibit virus entry and fusion, the various classes of RT inhibitors (NRTIs, NtRTIs and NNRTIs) and integration inhibitors. The primary benefit of a

combination microbicide would be that the barrier against infection is enhanced to cover additional mechanisms of antiviral activity against the same target or against multiple targets of infection. By incorporating two active ingredients with different mechanism of action, the microbicide can target different stages in the replication cycle of HIV. An example would be using a CCR5-antagonist such as maraviroc, which would be ineffective against a CXCR4-tropic virus, but in combination with an NNRTI would make the microbicide more effective than maraviroc alone (Garg *et al.* 2009). Balzarini *et al.* showed in 2012 that combining tenofovir with other ARVs resulted in synergistic to additive antiviral activities *in vitro*. For example, tenofovir combined with binding and fusion inhibitors, specifically carbohydrate-binding agents (CBAs), showed significant synergistic antiviral effects (Balzarini and Schols. 2012). The CBAs have been shown to target components of the gp120 transmembrane glycoprotein on the envelope of HIV, making them potent fusion inhibitors (Balzarini and Schols. 2012). It was also shown that CBAs can prevent syncytium formation, which causes spread of infection to uninfected CD4+ lymphocytes (Balzarini and Schols. 2012). Synergistic and additive interactions of combinations of ARVs also provides benefits such as necessitating lower doses of each ARV used in the combination than would be used in monotherapy (Adams and Kashuba 2012). These lower doses decrease the potential for systemic exposure to the compounds, as well as decreasing potential adverse effects. One might also imagine that the use of highly potent anti-HIV compounds which target pre-integration events in replication with highly potent agents which target late stage events could also have significant impact on virus transmission as long as the initially infected cells have no means by which to spread the acute infection. For example, RT inhibitors used in

combination with protease inhibitors, which act to prevent the formation of mature HIV constituent proteins could be envisioned to yield long term suppression of HIV and eventual, if not immediate, inhibition of transmission. If a virus cannot infect and initiate a productive, spreading infection, then eventual sterilization should occur.

Another benefit to combination microbicides would be the influence on the development of resistant viruses because a virus is less likely to be resistant to two different classes of inhibitors than it is to a single class inhibitor and thus, the barrier to initial infection by resistant virus would be even stronger (Garg *et al*. 2009). It is well known that the use of a combination of ARVs would decrease the rapid resistance development seen in monotherapy during therapy of infected patients. For example, Adams *et al.* in 2012 reported that *in vitro*, tenofovir and dapivirine in combination showed synergistic effects against the Y181C mutation which commonly emerge in the presence of NNRTIs, which they suggest supports the hypothesis that combination microbicides have activity against resistant viruses and therefor offer better chemical barrier protection. This is especially true regarding the Y181C mutation, which is known to be easily transmitted because the mutation impacts viral fitness very little (Adams and Kashuba 2012).

The effect of candidate microbicides against common drug resistant viruses was tested in the ICCA. In these assays, both IQP-0528 and dapivirine were tested against viruses harboring the common NNRTI resistance engendering mutations Y181C, K103N and A17 (K103N +Y181C). These viruses were chosen because they appear readily upon use of NNRTIs following HIV therapy and they are naturally occurring and preexisting in wild type populations as well as populations from patients previously treated with

NNRTIs. The results of the assays show that both IQP-0528 and dapivirine require greater concentrations in order to sterilize cultures when the infecting virus is an NNRTI-resistant virus. Although more drug was needed, the wide therapeutic index of these NNRTIs still allow for sterilizing concentrations to be achieved against the resistant viruses, with the exception of the A17 virus. The results obtained suggest that the ICCA was able to segregate these two NNRTI inhibitors by activity against resistant viruses and indicated that dapivirine was more active against the Y181C and K103N mutations than IQP-0528, but both compounds perform poorly against A17. The data generated from the ICCA make sense when considering NNRTIs have limited ability to function against NNRTI specific resistant viruses and how these mutations effect the binding ability of the NNRTIs. The ICCA showed decreased activity and increased sterilization concentrations for IQP-0528 for all mutants ranging from 10 to 200- fold less activity depending on the mutant. A17 showed the greatest decrease in activity due to both K103N and Y181C being present. In performing these assays, the reproducibility of the ICCA with all sterilizing concentrations were determined to be within 2-fold of each other, which is within the acceptable range for *in vitro* assays.

Resistance development to NNRTIs can be unique in that a single nucleotide change to the allosteric binding site can cause a dramatic effect on the activity of the NNRTI and allow for rapid resistance development (Bacheler *et al.* 2000). Many of the mutations selected after exposure to NNRTIs are shown in structural models to be clustered in and around the site on the RT where NNRTIs bind, known as the NNRTI binding pocket (Bacheler *et al.* 2000). The specific mutations listed above were chosen because they are some of the most commonly selected for mutations when NNRTIs are

used for treatment and are critical to the efficacy of IQP-0528. Even low frequency of either K103N or Y181C mutations are associated with a significant increase of failure of monotherapy treatment (Wang *et al.* 2014). Wang *et al.* found in 2014 that the presence of the K103N and Y181C mutations showed no decrease in the fitness of HIV, and these mutants show similar replication ability to WT virus which makes them easily transmissible (Wang *et al.* 2014). As stated in the results section, the Y181C mutant is a mutation of a tyrosine residue to a cysteine at the 181 position in the palm domain of the p66 subunit of the RT. By altering this amino acid, structural models have shown decreased interaction of the 181 residue and the ARV, specifically nevirapine and efavirenz (Ibe and Sugiura 2011). Y181C is a common mutation seen when HIV is treated with NNRTIs, and in a large-scale analysis of drug resistant genotypes from clinical samples, the Y181C mutation has been found in 17% to 27% of clinical samples reported by Ibe *et al*. in 2011 (Ibe and Sugiura 2011). This mutation allows the RT to retain similar activity to what is seen in wild type RT, with only slightly decreased activity of the RNase H (Ibe and Sugiura 2011). K103N consists of mutation from a lysine to an asparagine at the 103 position on the palm domain of the p66 subunit of the RT. The change in this residue has been shown to change the arrangement of the NNRTI binding pocket by forming a new network of hydrogen bonds. These bonds cause a conformational change to the binding area into the "closed" position, prohibiting NNRTI entry (Ibe and Sugiura 2011). The K103N mutation also confers a slight decrease in RNase H activity but shows no difference in its binding affinity of nucleotides, so it shows little difference in replication efficiency compared to WT (Ibe and Sugiura 2011). In the same large-scale study of resistant clinical isolates mentioned above, it was

determined that a K103N mutation was seen in 43 to 57% of samples (Ibe and Sugiura. 2011). These results suggest that selection of combination products to form a chemical barrier to infection must take into account the prevalence of these resistant viruses in the population. Thus, NNRTIs will need to be used in combination with agents from different mechanistic classes to assure that NNRTI-resistant strains don't bypass the chemical barrier as readily.

Another important aspect of microbicide development that was simulated in the ICCA was the effect of pretreatment on the standard ICCA with HIV-1_{IIIB}. In this experiment, cultures were pretreated with various concentrations of IQP-058 to determine the effect on the sterilization concentration observed. These experiments showed that there was no benefit to pretreatment with IQP-0528 unless the concentration remaining in the cultures post pretreatment and post infection was greater than 0.1 µM, the determined sterilizing concentration. For example, cultures pretreated with up to 20 µM IQP-0528 showed no suppression of viral transmission after IQP-0528 was removed from the cultures. However, when cultures were pretreated with 5 µM IQP-0528 or greater and residual compound remained in the culture instead of being removed through washing the cells, the cultures showed suppression of virus transmission. This was due to the sterilizing concentration of IQP-0528 still being maintained in the cultures. Because IQP-0528 is freely diffusible through the cell membrane and already exists in its active state, pretreatment will have no effect on the sterilizing concentration. This freely diffusible characteristic of NNRTIs makes them ideal candidates for development into microbicide products such as vaginal rings, which release a constant concentration of inhibitor to prevent HIV infection and decrease systemic exposure overall (Baeten *et al*.

2016). It can also be hypothesized that use of a NRTI such as tenofovir or AZT would be benefit from pretreatment in the ICCA due to the lag time associated with metabolic activation of the compounds and the fact that after metabolism the compounds are retained inside the cell at high concentration. Concentrations of the active forms of AZT, tenofovir and other NRTIs would be able to reach sterilizing concentrations before the culture is challenged with virus. In the case of these compounds, pretreatment and removal of extracellular drug would not eliminate activity since the metabolized active compound remains resident inside the cell and cannot leach out due to the charges introduced by phosphorylation.

Products such as intravaginal rings are being developed and tested in the clinic to determine the advantages of ARV pretreatment and PrEP. The rationale for development of an ARV-containing intravaginal ring comes from the use of vaginal ring-based contraception which provides a sustained and controlled release of a contraceptive (Baeten *et al.* 2016). The ASPIRE clinical trial developed one of the first microbicide intravaginal rings containing the NNRTI dapivirine. In Phase I trials, biopsies of vaginal tissues obtained from women using the dapivirine ring were shown to be less susceptible to HIV infection *ex vivo* than tissues obtained from the placebo group (Baeten *et al.* 2016). It was concluded that the dapivirine vaginal ring was safe and also showed plasma concentrations of dapivirine 1,000- fold less in women using the ring than in those taking dapivirine orally (Baeten *et al*. 2016). In the Phase III, placebo-controlled study, vaginal rings containing 25 mg of dapivirine were given to participants with instructions to insert a new ring every 4 weeks. To determine adherence, dapivirine concentrations were measured in the plasma of the participants as well as dapivirine

concentrations remaining in the discarded vaginal rings. Specific thresholds of dapivirine concentrations present in the plasma and remaining in the ring were set so adherence level could be determined. The study concluded that use of a 25 mg dapivirine vaginal ring showed a 37% reduction in HIV infection compared to the placebo (Baeten *et al.* 2016). It was also observed that between the groups, there were no significant differences in acquired sexually transmitted infections and no significant differences in emergence of resistant viruses (Baeten *et al.* 2016). Although there was significant protection provided to those using the dapivirine ring, the study reports that this protection was still lower than anticipated (Baeten *et al.* 2016). This lower than anticipated result is likely due to non-adherence of participants, especially those younger than age 21, involved in the study (Baeten *et al.* 2016). Low adherence is thought to be caused by the age of the participants, the side effects of the product or because of safety and efficacy concerns of the participants (Baeten *et al.* 2016). Studies with tenofovir have shown that adherence to a regimen was increased after the safety and efficacy of the drug had already been shown, so there is hope of increased adherence in future studies (Baeten *et al*. 2016). These results suggest that the IVR delivers a constant concentration of antiretroviral product over the course of 4 weeks and if used correctly (high adherence) offers protection from virus transmission. The ICCA measures the same constant concentration to define the sterilizing concentration and thus we believe that the calculation of intracellular drug concentration in the tissues of protected women using the IVR should relate to the sterilizing concentration of the drug in the ICCA. Our results suggest that the correlation between *in vitro* ICCA results and *in vivo* suppression is approximately 10 to 100-fold which suggests that the ICCA results are similar to *in vivo* protection results. Additional

studies would need to be performed to see if this holds true across trials with all microbicide products.

In contrast to pretreatment with IQP-0528, the effect of addition of IQP-0528 post infection was also tested, which can be used to determine the effect of using a microbicide product after an exposure event. These results determined that adding the known sterilizing concentration of IQP-0528 to an infected culture as little as 2 hours post infection is not adequate to prevent virus transmission. The addition of 0.1 μM IQP-0528 at 2 to 4 hours post infection showed suppression of virus transmission, but virus emerged after IQP-0528 had been removed from the cultures within 7 days, suggesting IQP-0528 was not able to act in time to prevent initial infection events from occurring. However, increasing the concentration of IQP-0528 two-fold in the ICCA demonstrated that IQP-0528 was able to suppress transmission when added within 2 hours post infection. It can be inferred from this data that if a sufficient dose (i.e. greater than the sterilizing concentration) is given post infection, spreading infections can be suppressed and a culture can be sterilized even after infection has initiated. Delayed addition ICCA experiments were not performed in the presence of different classes of compounds, but it can be assumed that different classes of compounds would react differently with delayed addition based on their mechanism of action. For example, NRTI inhibitors would most likely work less efficaciously in this variation of the ICCA due to the delay already resulting from the phosphorylation events required for active states to be reached. This variation to the ICCA method could be used to determine the effects of missing a dose or doses of a microbicide and what low adherence would mean to the sterilizing concentration of an ARV.

Many benefits of using the ICCA have been determined from the data generated in these experiments. Based on several studies performed with IQP-0528 and HIV-1_{IIIB} it has been shown that the ICCA is reproduceable, yielding a sterilizing concentration for IQP-0528 of 0.1 μM. Against resistant viruses, sterilizing concentration results from duplicate assays were within 2-fold, which is well within the acceptable range of error for most *in vitro* assays. Dapivirine also showed consistent, reproduceable sterilizing concentrations, and showed sterilizing concentrations in duplicate experiments against resistant viruses to be within 4-fold, also within the acceptable standards for *in vitro* assays. Results obtained in experiments with tenofovir also yielded highly reproduceable data with sterilizing concentrations determined the be greater than 120 μM in duplicate assays. Assays evaluating compound pretreatment and delayed addition of compounds, as well as MOI variability also showed consistent sterilizing concentrations of IQP-0528 within 4-fold in replicate assays. The ICCA is robust in its ability to rank order compounds based on activity in the assay and thus it could be used to prioritize microbicide products for development and clinical evaluations.

Although there were many benefits to the ICCA, including reproducibility and robustness, there are limitations associated with the ICCA that need further evaluation. One limitation observed in the ICCA was the requirement for PCR amplification, the RT activity assay and SFU counts taken for all samples. In some experiments performed, there would be (1) RT activity with no corresponding syncytia presence, (2) Syncytia presence with no corresponding RT activity or, (3) Syncytia and RT activity but no PCR product. These observations were mostly seen in the presence of resistant viruses. This may mean that some or all of these methods may not be sensitive enough to detect small,

low levels of virus in the infected cells in the cultures. Two additional methods that could be used are nested PCR amplification and p24 ELISA to increase the sensitivity and specificity to determine infection; however, utilizing these methods to obtain increased sensitivity for detection is associated with increased costs and labor. Another limitation of the ICCA was inconsistency with the days associated with viral break through. For example, in one ICCA performed with dapivirine and HIV-1_{NL4-3}, all cultures became infected before day 12 of the ICCA and in the second ICCA all cultures became infected only after compound was removed on day 12. These two assays both returned the same results, they just occurred at different time points within the ICCA. Typical for *in vitro* antiviral assays, these effects are likely associated with breakpoints in antiviral activity. Greatest variation in antiviral results are typically seen around the 50% inhibitory value and around the 100% inhibitory value. The ICCA is trying to quantify the 100% inhibitory concentration and thus variability would be expected to be seen. One of the biggest limitations of the ICCA would be that the results we are seeing do not necessarily reflect the complexity of the biological systems they are trying to mimic. The ICCA is performed with a limited number of cells and using a single cell type unlike in an environment where a microbicide would need to work. A vaginal environment would contain many different cell types, layers of tissue to penetrate and a major increase in the number of cells present at the site of infection. However, this is also a limitation associated with all *in vitro* assays. The ICCA is also limited in that only the active ingredient in a microbicide product can be used in the assay. Testing with formulated microbicides is difficult due to viscosity and cellular toxicity issues. Another limitation to the ICCA, and essentially all *in vitro* antiviral assays, is that pharmacokinetics are not

approximated and thus the *in vitro* results are obtained with a constant high level of drug bathing the target cells whereas *in vivo,* the exposure would experience peaks and troughs in drug concentration and variable cellular uptake depending on the cell type and location.

To combat some of limitations of the ICCA, more work needs to be performed to develop the ICCA to its full potential. Future experiments will include testing in vaginal and seminal fluids to make the ICCA more biologically relevant. The biological relevance could also be improved by adapting the ICCA to be used with cells more relevant to the vaginal and rectal environments to determine the changes in sterilization concentrations of ARVs in primary cells, as well as different relevant cell types. Peripheral blood mononuclear cells (PBMCs) do not form syncytium when infected with HIV so using the SFU assay to determine the percentage of infected cells in a population would be ineffective. This would alter the endpoint of the standard ICCA and put more emphasis on the RT assay and PCR results for infectivity determination of the cultures. Testing clinical virus isolates in the ICCA would also be beneficial and provide more real-world insight to dosages needed to combat non-laboratory adapted HIV strains; however, many clinical isolates do not infect CEM-SS cells as they are mostly CCR5 tropic. If the ICCA could be optimized for use with different cell types such as PBMCs, clinical isolates could be examined. Testing additional combinations of inhibitors have been and will continue to be performed to determine if there are synergistic or antagonistic antiviral interactions, and potentially develop new avenues for microbicide combination products. A broader range of inhibitors will also be tested to see if the robustness of the ICCA can also be used to segregate other classes of inhibitors besides

ones targeted to the RT. More complex but important testing might involve *ex vivo* tissue explant samples to determine if the sterilizing concentration produced by the ICCA are relevant and what dosing regimens would be needed to achieve these sterilizing concentrations necessary at the tissue level. We have performed some preliminary testing in nonhuman primates as a component of a NIH grant and the achieved tissue drug concentration data can be correlated with the ICCA-defined concentrations for IQP-0528, dapivirine and tenofovir.

In conclusion, the ICCA can be used to determine the sterilization concentration of ARVs being developed as microbicides using an *in vitro* assay which has been adapted to mimic the sexual transmission of HIV. The ICCA has been shown to work with a variety of laboratory strains of HIV-1, as well as have the ability to be used to determine the effects of drug pretreatment (PrEP) and drug post-treatment (applying a microbicide after exposure or compounds with poor or delayed uptake) and their effect on the sterilizing concentration. The ICCA is more predictive than the MTSA in that all of the cells in the culture are retained until cell densities became too high, unlike in the MTSA where 80% of the culture is removed every 3 days, potentially removing infected cells early in the assays, decreasing the sensitivity. The ICCA will benefit the microbicide field by facilitating more predictive dosing strategies and in providing a rationale for dosing with ARVs instead of dosing with the highest amount of ARV tolerated by the environment where the microbicide will be used, which is a strategy that can lead to systemic toxicity as well as rapid emergence of resistant viruses. The ICCA may also provide a link from *in vitro* to *in vivo* testing, decreasing the reliance on predictive animal models. With more investigation, the ICCA can be a more predictive *in vitro* assay used

to predict dosing concentrations of ARVs needed in microbicide products to prevent transmission of HIV and sterilize cultures compared to *in vitro* assays currently being used in the microbicide testing algorithm.

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