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Resumen

Actualmente existe una necesidad, no alcanzada todavía de proteger a las mujeres contra las enfermedades de transmisión sexual y el embarazo no deseado. En 2013 se produjeron 2,1 millones de nuevas infecciones por VIH y 1,5 millones de muertes debidas al VIH/SIDA en todo el mundo. Los casos reportados en mujeres representan aproximadamente la mitad de las infecciones por VIH, la mayoría de las cuales se producen por transmisión sexual, tanto por vía vaginal como anal. Existen evidencias sólidas que indican que la infección con Herpes Simplex humano tipo 2 (VHS-2) está asociada con un aumento de la carga viral de VIH y, por lo tanto, con un aumento del riesgo de transmisión. El principal gel microbicida (PC-1005) desarrollado en nuestra Institución, Population Council, que contiene 50 mM del inhibidor no nucleósido de la transcriptasa reversa MIV-150 y 14 mM de acetato de zinc dihidrato en carragenina, protege contra la infección vaginal y rectal del virus de la inmunodeficiencia humana/simia (VIHS-RT) *in vivo*. Además, el PC-1005 resulta prometedor como agente microbicida de amplio espectro contra el VHS-2 y el virus del papiloma humano (VPH) tipo 16, 18 y 45 en pruebas con modelos murinos y *in vitro*. Teniendo en cuenta estas observaciones hemos desarrollado un anillo intravaginal (AIV) de prevención polivalente que libera PC-1005 para proteger a las mujeres contra el VIH, VHS-2 y también el VPH. Este AIV además libera Levonogetsrel con el propósito de proteger también contra el embarazo no deseado que es una de las causas más frecuentes de mortalidad materna en países en desarrollo.

En este estudio demostramos, usando un modelos de explantes simios y humanos, que el PC-1005 es seguro y posee una actividad potente contra el VIHS-RT, VIH y VHS-2. Nuestros datos además endorsan el desarrollo del gel PC-1005 como microbiocida de amplio espectro. Aquí también demostramos que el prototipo de AIV que libera, además de PC-1005, leovonorgestrel, es efectivo contra el VIHS-RT en explantes genitales de macacos. Este estudio demuestra que el AIV es un candidato viable para prevenir VIH, VHS-2, VPH y embarazos no deseados y así mejorar la salud sexual de millones de mujeres en todo

el mundo.

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Indice

ABBREVIATIONS

3TC	lamivudine
Acy	acyclovir
AIDS	acquired immune deficiency syndrome
API	active pharmaceutical ingredient
CAPRISA	Centre for the AIDS Program of Research in South Africa
CCR5	C-C chemokine receptor type 5
CD3	cluster of differentiation 3
CD38	cluster of differentiation 38
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CG	carrageenan
CVLs	cervico-vaginal lavages
CXCR-4	C-X-C chemokine receptor type 4
DCs	dendritic cells
DRMs	multiple drug resistance mutations
EVA	ethylene vinyl acetate
FBS	fetal Bovine serum
FGT	female genital tract
gp120	viral envelope glycoprotein GP120
HEC	hydrocellulose
HIV	human immunodeficiency virus
HNP	human neutrophil polypeptide
HPV	Human papilloma virus
HSV-2	Herpes Simplex Virus type 2
IL-2	interleukin 2
IL-2	interleukin 2
IVR	intra vaginal ring
LCMS/MS	liquid chromatography–mass spectrometry

LLOQ	lower Limit of quantification
LNG	levonorgestrel
mAbs	monoclonal antibodies
MPT	multipurpose prevention technologies
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MZCL	MIV-150-zinc acetate dehydrate-carrageenan-levonorgestrel
NDRI	National Disease Research Interchange
NF-κB	nuclear transcription factor κB
NNRTI	non-nucleoside reverse transcriptase inhibitor
PBMCs	peripheral blood mononuclear cells
PD	pharmacodynamics
PK	pharmacokinetics
PHA	phytohaemagglutinin
PrEP	pre-exposure prophylaxis
qPCR	quantitative polymerase chain reaction
qRT-PCR	reverse transcription qPCR
RA	retinoic acid
RIA	radioimmunoassay
RM	Rhesus macaque
SHIV-RT	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus
SLPI	secretory leukoprotease inhibitor
STIs	sexually transmitted infections
$TCID_{50}$	median tissue culture infective dose
TFV	tenofovir
TNPRC	Tulane National Primate Research Center
VF	vaginal fluid
ZA	zinc acetate
α4β7	gut-specific homing Integrin α4β7

SP	seminal plasma

I. INTRODUCTION

HIV Epidemiology

During sexual intercourse, Human Immunodeficiency Virus (HIV) transmission from male to female is more efficient than from female to male, thereby making women a disproportionately more susceptible population to HIV infection (Nicolosi, Correa Leite et al. 1994). Although the risk of vaginal HIV transmission is relatively infrequent when considering the risk of infection per coital act (Dosekun and Fox 2010) and estimated to be 1 in 1,250 exposures (Boily, Baggaley et al. 2009) , risk of infection can be greatly influenced by the infectiousness of sexual partner and susceptibility of the exposed individual (Dosekun and Fox 2010). It has been estimated that 76% of young people (aged 15–24 years) living worldwide with HIV are female with vaginal intercourse remaining the most prevalent route of infection (Epidemic.UNAIDS. 2010). In addition, the Joint United Nations Programme on HIV/AIDS (UNAIDS) reported that around 36.7 million [34.0 million–39.8 million] people globally were living with HIV in 2015, with 2.1 million new HIV infections. Despite the fact that AIDS-related death have fallen 45% since the peak of the epidemic, in 2015 1.1 million [940 000–1.3 million] people died from AIDS-related causes (UNAIDS 2016). Tuberculosis remains the leading cause of death among people living with HIV, accounting for around one in three AIDS-related deaths. As of December 2015, only 17 million people living with HIV worldwide were accessing antiretroviral therapy, 46% [43–50%] of all adults living with HIV were accessing treatment in 2015 with regions such as the Middle East and North Africa having only 16% [12–24%] access to anti-retroviral therapy (UNAIDS 2016). In western, eastern, central and southern Africa, women account for around 60% of HIV infections (Abdool Karim, Sibeko et al. 2010; UNAIDS 2016).

Events in HIV mucosal transmission

Vaginal and cervical epithelia are most likely the primary sites where HIV from male semen and cells from the female genital tract first encounter. Interestingly, although most studies on mucosal HIV transmission have focused on cell-free virions (Bomsel and David 2002), all genital fluids that transmit HIV such as seminal plasma and cervicovaginal secretions also carry HIV-infected cells (Anderson, Politch et al. 2010). HIV gains entry into the body mainly during sexual intercourse by crossing epithelial barriers covering the mucosal surfaces of the female genital tract (FGT) as well as the anal/rectal epithelia (Tebit, Ndembi et al. 2012). The FGT is protected from virus penetration by a multilayered squamous and columnar epithelium in the ectocervix/vaginal vault and endocervix, respectively. (Anderson, Marathe et al. 2014). In order for HIV transmission to occur, infectious virions or infected cells from the donor must cross these barriers to find a susceptible cell in the host.

HIV can penetrate the squamous epithelium in the FGT where target cells reside, by loss of tissue integrity as a result of ulcerative genital infections, abrasions that occur during intercourse (Carias, McCoombe et al. 2013) or possibly transcytosis (Bobardt, Chatterji et al. 2007). Furthermore, HIV entry is dependent on binding to its principal receptor CD4 , which triggers a conformational change in the viral envelope glycoprotein GP120 (gp120) and subsequent engagement with co receptors C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR-4) at mucosal sites which, in turn, leads to fusion between viral and cellular membranes. HIV infects particularly $CD4^+$ $CCR5^+$ T-cells that are HIV-specific or activated (Douek, Brenchley et al. 2002). Of note, the chemokine receptor CCR5 is the predominant fusion cofactor for the most transmitted HIV-1 strains (Lederman, Penn-Nicholson et al. 2006). Moreover, $CD4^+$ T-cells expressing α4β7 or α4β1 are highly susceptible cells in the FGT compartment (Joag, McKinnon et al. 2016). The main cellular $CD4^+$ target cells are $CD4^+$ lymphocytes, $CD4^+$ cells form the macrophage lineage and dendritic cells (DCs). DCs efficiently capture HIV and transmit captured or newly produced viral particles to T-cells (Turville, Aravantinou et al. 2008) where the DC-T cell communication drives robust virus replication (Derby, Martinelli et al. 2011). The retrovirus genus to which HIV-1 and SIV belong was long ago named *Lentivirinae* based on the long incubation period and slow clinical course of the disease that typify the infection. However it is now clear from the SIV Rhesus macaque (RM) model, that local events critical to stablish systemic infection, take place very quickly in the early stages of lentivirus infections. Within hours of infection, HIV establishes a founder population of infected cells (Zhang, Wietgrefe et al. 2004; Miller, Li et al. 2005) . This population expands locally during the first week of infection generating a viral pool that establishes a self-propagating systemic infection in the secondary lymphoid organs (Miller, Li et al. 2005). As virus has access to more susceptible target cells during the second week of infection, a significant increase in replication in the lymphatic tissues occurs. The peak of virus replication in blood and tissue happens during the second week after infection before declining to stable levels around four weeks post exposure (Haase 2010). The infected lymphatic tissues act as reservoirs for virus production and storage as well as harboring of proviruses in latently infected cells (Haase 1999). (Fig. 1)

Figure 1

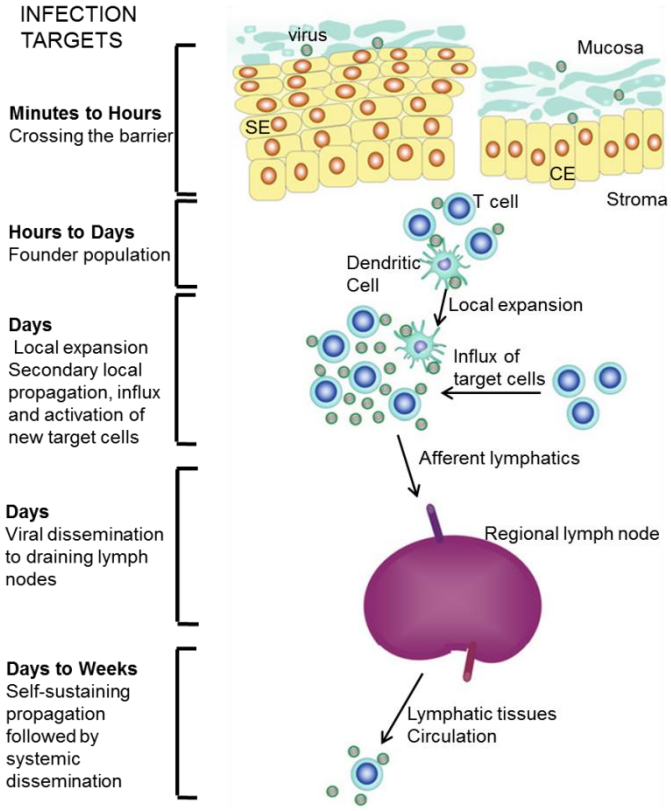


Fig.1- Time frame, sites and major events in vaginal transmission and the fast phase of lentivirus infection. The SIV rhesus macaque animal model provides a window through which to view early infection. Within hours, virus in the inoculum may gain access through breaks in the mucosal epithelial barrier to susceptible target cells. The small focal infected founder population is initially composed mainly of infected resting CD4 T cells lacking conventional markers of activation. The founder population expands locally in these 'resting' and in activated CD4 T cells. Local expansion is necessary to disseminate infection to the draining lymph node, and subsequently through the bloodstream to establish a self-propagating infection in secondary lymphoid organs. (Adapted from Haase 2010)

HIV and HSV-2 co infection

Pre-existing infection with other sexually transmitted infections (STIs) is a significant cofactor of HIV-1 transmission(Barnabas, Webb et al. 2011). Among STIs affecting HIV transmission and pathogenesis, non-curable STIs like Herpes Simplex Virus type 2 (HSV-2) and Human papilloma virus (HPV) deserve special attention. Epidemiological data show a link between the prevalence of HSV-2 and HIV-1(Weiss 2004; Lama, Lucchetti et al. 2006). While HSV-2 appears to make a woman more susceptible to HIV-1 infection the opposite also seems to be true: HIV-1 positive women have impaired mucosal immunity to fight an HSV-2 primary infection or even reactivation (Kaul, Kimani et

al. 2004; John, Keller et al. 2005; Rebbapragada, Wachihi et al. 2007). HIV-1 and HSV-2 also appear to have a synergistic relationship infection on a host viral shedding: HIV-1 infected women shed more HSV-2 virus and HSV-2 suppression decreases HIV-1 viral load in serum and HIV-1 RNA shedding in the genital tract (Rebbapragada, Wachihi et al. 2007; Baeten, Strick et al. 2008; Dunne, Whitehead et al. 2008; Lingappa, Baeten et al. 2010). Recent clinical studies with documented HSV-2 *prior* to HIV1 acquisition reported up to 3-fold and 7-fold increased risk of HIV-1 transmission with prevalent and incident HSV-2 infection, respectively (Glynn, Carael et al. 2001; Freeman, Weiss et al. 2006; Sobngwi-Tambekou, Taljaard et al. 2009; Barnabas, Webb et al. 2011; Barnabas and Celum 2012). In addition, infection with HSV-2 is associated with increased human immunodeficiency virus (HIV-1) acquisition (Corey 2007; Barnabas, Wasserheit et al. 2011). Globally, 536 million people were estimated to be infected with HSV-2 in 2003 and in populations where HSV-2 infection is highly prevalent, nearly half of the HIV-1 infections are attributed to HSV-2 co-infection (Wald and Link 2002; Looker, Garnett et al. 2008).

Mechanisms of enhanced susceptibility to HIV infection in the presence of HSV-2

The biological explanation on how HSV-2 increases a subject susceptibility to HIV-1 infection can be hypothesized based on several interacting mechanisms. The most conspicuous of it is the disruption on the genital epithelial barrier that would expose intraepithelial and lamina propia HIV-1 target cells (Van de Perre, Segondy et al. 2008) during the ulcerative phase. In addition, the presence of HSV-2 even in the absence of genital ulcers may recruit activated target cells for HIV-1 (Rebbapragada, Wachihi et al. 2007; Zhu, Koelle et al. 2007; Zhu, Hladik et al. 2009). Exposure to HSV-2 *in vitro* causes a significant downregulation of the secretory leukoprotease inhibitor (SLPI) gene expression by human epithelial cells which persists even in the presence of the anti-HSV nucleoside analogue Acyclovir(Fakioglu, Wilson et al. 2008). SLPI, an enzyme present in vaginal fluids and semen, has been shown to inhibit HIV-1 infection *in vitro* and its downregulation, in turn, causes upregulation of nuclear transcription factor κB(NF-κB) mediated pro-inflammatory pathway(Fakioglu, Wilson et al. 2008). Furthermore, NF-κB is also known to induce HIV-1 replication (Al-Harthi, Spear et al. 1998; Amici, Belardo et al. 2001; Goodkin, Ting et al. 2003).Conversely, *in vitro* experiments demonstrated that cervicovaginal lavages of HIV-1 seronegative women have a potent anti HSV-2 activity, which correlates with the concentration soluble defensins, specifically human neutrophil polypeptide 1-3 (HNP 1-3)(John, Keller et al. 2005). This group also reported reduction of HNP 1-3 in HIV-1 positive women. Zhu et al. after analyzing cellular infiltrates from biopsies of HSV-2 lesions demonstrated $CD4^+$ $CCR5^+$ T-cells harboring greater concentrations of integrated HIV-1 DNA compared with control samples not infected with HSV-2 (Zhu, Hladik et al.

2009). A recent study carried out in cultures of cervical explants, showed greater levels of HIV-1 replication in HIV-1/HSV-2 infected ectocervical tissues compared with tissues infected with HIV-1 alone(Rollenhagen, Lathrop et al. 2014). In this study, the characterization of the molecular mechanism revealed early upregulation of HIV receptor $CD4^+$, and co-receptor $CCR5^+$ in HIV/HSV-2 infected tissues compared with tissues infected with either virus alone suggesting general mucosal immune activation. Faster $CD4^+$, $CCR5^+$ and $CD38^+$ mucosal depletion was observed in co-infected tissues compared to single HIV-1 infected ones(Rollenhagen, Lathrop et al. 2014). It has been demonstrated that HIV-1 replication is more efficient in activated ($CD4^+$ $CD38^+$ T cells) compared to resting $CD4^+$ T cells (Biancotto, Iglehart et al. 2008; Jaspan, Liebenberg et al. 2011; McKinnon and Kaul 2012). Rollenhagen et al. detected 10-fold higher levels of CD38 RNA expression in HIV/HSV-2 co infected tissues compared with tissues infected with HSV-2 or HIV-1 alone and similar to what happens to $CD4^+$ $CCR5^+$, $CD38^+$ RNA expression levels decreased by 3-fold in the co-infection scenario compared with single infection. Furthermore, studies in rhesus macaques (RM) showed that vaginal HSV-2 infection is associated with increased susceptibility to the simian/human immunodeficiency virus $SHIV_{SF162P3}$ and provided some insights into possible mechanisms of increased transmission(Goode, Truong et al. 2014). Specifically, frequency of vaginal $CD4^+$ T cells expressing high level of α4β7, a gut-homing integrin that binds gp120 (Arthos, Cicala et al. 2008) and facilitates HIV/SIV infection(Arthos, Cicala et al. 2008; Cicala, Martinelli et al. 2009; Kader, Wang et al. 2009; Ansari, Reimann et al. 2011; Martinelli, Tharinger et al. 2011; Martinelli, Veglia et al. 2013; Goode, Truong et al. 2014), is increased in HSV-2 infected rhesus macaques(Goode, Truong et al. 2014). An increase of $\alpha4\beta7^{high}$CD4+ T cells in rectal mucosa was also observed in rectal HSV-2 infection in RM (Martinelli, Veglia et al. 2013).

Unwanted pregnancies and unsafe abortions

Worldwide, one in five pregnancies (20 percent) ends in abortion, and one in 10 pregnancies ends in unsafe abortion(WHO 2012). An estimated 42 million abortions are performed each year; 20 million of them are outside the legal system and considered unsafe because they are performed by people who lack the necessary skills or in places that do not meet minimal medical standards, or both (WHO 2012). An estimated 358,000 girls and women die from pregnancy-related causes each year, almost all of them in the developing world (WHO 2012). About 47,000 of these deaths are due to unsafe abortion(Shah and Ahman 2010).

Globally, abortion-related deaths account for 13 percent of all pregnancy-related deaths, but the percentage can be much higher at country levels(WHO 2012). A year 2000 study estimated that unsafe abortions were responsible for nearly one-third of maternal deaths in West Africa, and WHO

reports that in the countries of sub-Saharan Africa unsafe abortions are responsible for as much as 50 percent of maternal deaths(Bureau 2011).

Microbicides and Multipurpose Prevention Technologies (MPTs)

For women, the primary sexual and reproductive health risks of unprotected sex are unintended pregnancy and a variety of sexually transmitted infections (STIs), including HIV. Male and female condoms are the only currently available products that provide simultaneous protection against these risks, but acceptability (and cost issues, for female condoms) has constrained their widespread use (Peters, Jansen et al. 2010; Reynolds, Luseno et al. 2012). Other methods available to prevent HIV transmission are male circumcision and behavioral interventions but data suggest they are insufficient to protect women. Among women in sub-Saharan Africa, one of the highest risk factors for acquiring HIV is being in an stable long-term relationship where condom use is low (Shattock and Solomon 2004). Several vaccine research programs aiming to prevent HIV-1 and HSV-2 transmission are ongoing. However, so far only HPV prophylactic vaccines have been notably efficient. In recent years, a number of biomedical interventions have shown promise in HIV prevention. These include pre-exposure prophylaxis (PrEP), treatment as prevention, and microbicides. Microbicides are topical PrEP products, such as gels, capsules, tablets, films, and intravaginal rings (IVR). They are designed to be applied either around the time of sexual intercourse, used on a daily basis (gels and films), or to deliver product over a prolonged period of time (IVR). The premise is inhibition or blockade of the earliest steps in the infection process at the vaginal or rectal mucosa. Because microbicides are topical, higher local drug concentrations can be delivered to virally exposed surfaces without significant systemic exposure, thereby reducing the risk of long-term toxicity in healthy but at-risk individuals (Shattock and Rosenberg 2012).

The first trial where a microbicide against HIV proved to be effective against HIV acquisition was the Centre for the AIDS Program of Research in South Africa (CAPRISA) 004 study. The trial assessed the effectiveness and safety of a 1% vaginal gel formulation of tenofovir, a nucleoside reverse transcriptase inhibitor, for the prevention of HIV acquisition in women (Abdool Karim, Abdool Karim et al. 2010). The study showed that Tenofovir gel reduced HIV acquisition by an estimated 39% overall, and by 54% in women with high gel adherence. No increase in the overall adverse event rates was observed. There were no changes in viral load and no tenofovir resistance in HIV seroconverters (Abdool Karim, Abdool Karim et al. 2010).However, in light of the synergy between other STIs and HIV-1 acquisition, broad spectrum microbicides may more effectively limit HIV incidence (Rollenhagen, Lathrop et al. 2014). A recent study showed that in a subgroup CAPRISA 004, precoitally application of tenofovir gel reduced HSV-2 acquisition in women (Abdool Karim,

Abdool Karim et al. 2015). However, there is evidence that the enhancement of tissue susceptibility to HIV infection by HSV-2 co-infection, decreases the anti-HIV potency of tenofovir in an ectocervical explant model (Rollenhagen, Lathrop et al. 2014), stressing the importance of the development of a microbicide capable of protecting genital mucosa against HSV-2 as well as HIV-1. Multipurpose Prevention Technologies (MPTs) are new, all-in-one tools being developed to protect against HIV, other STIs, and, in some cases, unintended pregnancy. Much of the work on MPTs to date has focused on unintended pregnancy and HIV, but for the reasons aforementioned an ideal MPT should also ensure that other STIs are addressed as well. Therefore, the development of MPTs active against HIV-1, HSV-2 and HPV vaginally and rectally could significantly improve global public health (Houlihan, Larke et al. 2012; Malcolm, Boyd et al. 2014; Fernandez-Romero, Deal et al. 2015; Fernandez-Romero, Teleshova et al. 2015).

PC-1005 gel

The Population Council's MPT microbicide gel, PC-1005 (MZC), which contains the non-nucleoside reverse transcriptase inhibitor (NNRTI) MIV-150, Zinc acetate (ZA) and Carrageenan (CG) has a broad anti-HIV activity independent of the clade, tropism or phenotype and against multi drug resistant strains and clones containing multiple drug resistance mutations (DRMs) in cell-based assays (Kizima, Rodriguez et al. 2014). PC-1005 protects macaques against simian-human immunodeficiency virus (SHIV-RT) and partially reduces HSV-2 infection when applied vaginally up to 8 h before single high dose SHIV-RT or SHIV-RT/HSV-2 co-challenge (Kenney, Aravantinou et al. 2011; Kenney, Singer et al. 2012; Hsu, Aravantinou et al. 2014). In a repeated low dosing SHIV-RT/HSV-2 co-challenge model, PC-1005 and PC-525 (CG) gel significantly reduce the frequency and level of HSV-2 shedding (Kenney, Derby et al. 2014). Furthermore, PC-1005 inhibits vaginal and anorectal HSV-2 infection of mice (Kizima, Rodriguez et al. 2014; Jean-Pierre, Barnable et al. 2016). Importantly, the anti-HIV-1 and anti-HSV-2 activity of the gel is unaffected in the presence of human seminal plasma (SP) (Kenney et al., in preparation). We recently demonstrated that PC-1005 is active against cell-free and cell-associated SHIV-RT infection of macaque vaginal and cervical explants *ex vivo* (Barnable, Calenda et al. 2014; Barnable, Calenda et al. 2015; Jean-Pierre, Barnable et al. 2016) and its activity is not affected by SP (Barnable, Calenda et al. 2014). Furthermore, PC-1005 gel provides CG-mediated activity against HPV and this activity is also not compromised in the presence of SP (Rodriguez, Kleinbeck et al. 2014; Fernandez-Romero, Deal et al. 2015). The first Phase 1 clinical trial of PC-1005 demonstrated that vaginal administration of the gel is safe, well tolerated and leads to low MIV-150 absorption (Friedland, Hoesley et al. 2016)

MZCL intravaginal ring

To address the worldwide problem of unintended pregnancies, unsafe abortions and mortality at child birth while still protecting against STIs, the Population Council also developed a prototype IVR that releases the MIV-150, Zinc acetate dihydrate, Carrageenan, and the contraceptive Levonorgestrel (MZCL). Furthermore IVRs may overcome the issue of poor adherence in clinical trials that has overshadowed the success of microbicide gels in the HIV PreP field (a similar approach is being tested in a phase 1 trial with a TFV LNG IVR (Clark, Clark et al. 2014)). Unlike other multipurpose prevention technology (MPT) IVRs in development, dapivirine/LNG (HIV/unintended pregnancy), vicriviroc/progestin (HIV/unintended pregnancy) and tenofovir/LNG (HIV/HSV-2/unintended pregnancy)(Friend, Clark et al. 2013; Harrison, Hemmerling et al. 2013; IMPT 2015) , our MZCL IVR contains antiviral agents targeting HIV-1 (MIV-150, and zinc acetate), HSV-2 (CG and CG with zinc acetate), and HPV (CG). Although there is a concern for the potential emergence of drug-resistant HIV following use of microbicides containing antiretroviral drugs in infected individuals who are either unaware of their HIV infection status or who are aware but still choose to use the microbicide, the MIV-150 and ZA combination provides anti-HIV-1 activity, increasing potency and minimizing drug resistance issues (Kenney, Aravantinou et al. 2011). In agreement with these data, topical delivery of MIV-150 from IVR for 56d in rhesus macaques led to emergence of one drug resistance mutation (E138K, associated with intermediate or low-level resistance to NNRTIs) in 2 out of 6 animals (430 HIV-1 reverse transcriptase sequences analyzed total; 0.46%), indicating a low probability for the emergence of drug resistance mutations after sustained topical delivery of MIV-150 (Hsu, Keele et al. 2014). LNG is a licensed contraceptive that has been approved for non-oral controlled-release delivery via implants and intrauterine systems and it is included in several microbicide/contraceptive IVRs under development (Thurman, Clark et al. 2013). It has been previously shown that prototype core-matrix macaque-sized MZC and MZCL IVRs released all APIs *in vitro* for at least 90d and *in vivo* for at least 28d (Ugaonkar, Wesenberg et al. 2015). Moreover, MIV-150 in blood and vaginal fluids (VF) and LNG in blood reached levels associated with efficacy (protection against vaginal SHIV-RT challenge and contraceptive activity)(Nilsson, Lahteenmaki et al. 1980; Barbosa, Bakos et al. 1990; Luukkainen and Toivonen 1995; Singer, Mawson et al. 2012; Hsu, Keele et al. 2014). In addition, CG was also detected in VF (Ugaonkar, Wesenberg et al. 2015).

Tissue explants as a tool for pre-clinical evaluation of microbicides.

Preclinical laboratory assays and animal models used to evaluate products for activity against HIV-1 have been poor predictors of effectiveness in HIV prevention. The majority the preclinical

efficacy and safety testing of microbicide products has been done using a variety of in vitro assays that use primary immune cells and cell lines. There are several algorithms in use that vary in the specific assays to characterize the performance of the drugs under different environmental conditions (Dezzutti, James et al. 2004; Lard-Whiteford, Matecka et al. 2004; Lackman-Smith, Osterling et al. 2008). Until this past decade, most algorithms have not incorporated *ex vivo* mucosal tissue which includes cervical, vaginal, colonic, penile, foreskin, and tonsil. Non-polarized Rhesus macaque and human tissues are used to address specific questions regarding product safety, efficacy, and drug localization (Dezzutti, Uranker et al. 2013; Barnable, Calenda et al. 2014; Barnable, Calenda et al. 2015). Non-polarized tissue is typically composed of small pieces (2 or 3 mm^3) of tissue that are cut from the larger piece using a scalpel, the mucosa is retained, but the muscle layer is trimmed off (Lard-Whiteford, Matecka et al. 2004; Fletcher, Elliott et al. 2006; Richardson-Harman, Lackman-Smith et al. 2009). The tissue is submerged in medium that contains drug with or without HIV or HSV-2. This creates the "worst-case scenario" by allowing virus access to targets in the lamina propria independent of traversing the epithelium(Dezzutti and Hladik 2013). Using this model active pharmaceutical ingredients (APIs) have been tested to determine safety in tissue and efficacy against HIV-1 and HSV-2 infection (Fletcher, Kiselyeva et al. 2005; Fletcher, Wallace et al. 2006; Fletcher, Harman et al. 2009; Andrei, Lisco et al. 2011; Barnable, Calenda et al. 2014; Barnable, Calenda et al. 2015). The potency of several APIs has been tested in non-polarized tissue demonstrating that several log_{10} more drug is needed to block HIV-1 infection of tissue than what is found for traditional *in vitro* assays such as cell lines (Fischetti, Barry et al. 2009; Fletcher, Harman et al. 2009; Herrera, Cranage et al. 2011). Preclinical testing in tissue explant of products used in Phase III efficacy trials showed these compounds either were not effective against HIV-1 or were toxic infection and thus led to increased HIV-1 infection in the clinical trial subjects (Dezzutti, James et al. 2004; Beer, Doncel et al. 2006; Fletcher, Harman et al. 2009). Finally, the use of mucosal tissue to date has not only provided critical information regarding the feasibility of using antiretroviral drugs for HIV-1 prevention, but also demonstrated some predictability of clinical success(Rohan, Moncla et al. 2010)

II. OBJECTIVES

1. To assess the activity of PC-1005 (MZC) against SHIV-RT and HSV-2 in macaque vaginal and rectal mucosa and evaluate the in vitro activity of PC-1005 and cervicovaginal lavages (CVLs) collected at 4h and 24 h post PC-1005 application in Phase 1 trial against single HIV-1 and HIV-1/HSV-2 co-infection in human ectocervical explants.

   a) Establish and optimize human cervical and macaque vaginal, cervical and rectal explant co-infection models for microbicide testing.

   b) Design and evaluate a quantitative PCR (qPCR) and Reverse Transcription qPCR (qRT-PCR) to monitor accumulation of HSV-2 and HIV-1/SIV viral copies in the supernatant of the explant cultures as an alternative to traditional plaque (HSV-2) assay and p24 and p27 ELISAs (HIV-1/SIV) (Abner, Guenthner et al. 2005; Richardson-Harman, Mauck et al. 2012; Barnable, Calenda et al. 2014) respectively.

2. To evaluate the anti-SHIV-RT activity of MZCL IVR in genital mucosa by

   a) Determining whether LNG interferes with MIV-150's anti-HIV activity.

   b) Assessing the activity of vaginal fluids (VF) from macaques carrying MZC or MZCL IVRs (available from previously completed studies) (Ugaonkar, Wesenberg et al. 2015)

   c) Studying whether vaginal and cervical mucosae from animals administered MZCL IVRs (vs. LNG IVRs) are protected against SHIV-RT after IVR insertion and relate the effects to MIV-150 concentrations in tissues, VF and plasma.

III. MATERIALS AND METHODS

1. Macaques studies

1.1 Ethics statement

Adult female Chinese and Indian rhesus macaques (Macaca mulatta) were housed and cared for in compliance with the regulations under the Animal Welfare Act (U.S. Department of Agriculture), the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services 1985), at Tulane National Primate Research Center (TNPRC; Covington, LA). All studies were approved by the Animal Care and Use Committee of the TNPRC (OLAW assurance #A4499-01). Animals were monitored continuously by veterinarians to ensure their welfare. Animals in this study were socially housed unless restricted by study design. Housing restrictions were scientifically justified and approved by the IACUC as part of protocol review. Animals were housed indoors in climate controlled conditions with a 12/12 light/dark cycle. Animals in this study were fed commercially prepared monkey chow twice daily. Supplemental foods were provided in the form of fruit, vegetables, and foraging treats as part of the TNPRC environmental enrichment program. Water was available at all times through an automatic watering system. The TNPRC environmental enrichment program is reviewed and approved by the IACUC semiannually. Extensive efforts are made to find compatible pairs for every study group, with additional environmental enrichment of housing space through a variety of food supplements and physical complexity of the environment. A team of 11 behavioral scientists monitors the wellbeing of the animals and provides direct support to minimize stress during the study period. Anesthesia was administered prior to and during all procedures, and analgesics were provided afterwards as previously described (Kenney, Aravantinou et al. 2011; Singer, Mawson et al. 2012). All biopsy procedures were performed by Board Certified veterinarians (American College of Laboratory Animal Medicine). Biopsies were collected not more often than every 4 weeks. Veterinarians at the TNPRC Division of Veterinary Medicine have established procedures to minimize pain and distress through several means. One animal got sick during in vivo IVR (APIs concentrations and tissue PD study) and was euthanized. Vaginal necropsy tissues derived from additional animals were available from separate concurrent studies. The animals were euthanized using methods consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia. The animal is anesthetized with tiletamine/zolazepam (each at 4 mg/kg IM) and given buprenorphine (0.01 mg/kg IM) followed by an overdose of pentobarbital sodium. Death is confirmed by auscultation of the heart and pupillary dilation. TNPRC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC#000594).

1.2 PC-1005 activity against SHIV-RT and HSV-2 in macaque tissues.

1.2.1 PC-1005 gel anti SHIV-RT and HSV-2 activity: Chinese and Indian rhesus macaques (Macaca mulatta) were utilized.

1.2.2 MIV-150 and LNG toxicity, anti-SHIV-RT activity in vitro and ex vivo (VF PD). Necropsy vaginal tissue specimens were available from n=16 SHIV or SHIV/HSV-2 exposed, uninfected and infected Chinese and Indian macaques. Six of these macaques were Herpes B positive. Vaginal biopsies were collected from n=11 Naïve, SHIV exposed, uninfected and infected Chinese and Indian macaques. One of these animals was Herpes B positive. Animal ages ranged from 5.7-22 years old and their weights ranged from 4.3-12 kg. VF (swabs) were available from SHIV/HSV-2 exposed, uninfected and infected Chinese and Indian macaques with MZC (n=11) and MZCL (n=9) IVRs inserted for 21 or 28d in previously completed studies (Ugaonkar, Wesenberg et al. 2015). Animal ages ranged from 5.25 - 13.15 years old and their weights ranged from 5.5 - 14 kg.

1.2.3 In vivo IVR study (API concentrations and tissue PD). Vaginal and cervical biopsies, blood samples and VF (swabs) were derived from n=10 Naïve Indian macaques. Animals were inserted MZCL (n=5) and LNG (n=5) IVRs for 24h. Animal ages ranged from 7 - 12.7 years old and their weights ranged from 6 - 12 kg.

2 Human studies

2.1 Ethical review .The Phase 1 trial (Population Council protocol #558) was approved by the Institutional Review Boards of the Population Council and University of Alabama Birmingham School of Medicine. All subjects provided written informed consent for specimen collection, storage, and use in this study.

2.2 Human ectocervical tissues without gross pathological changes were obtained from routine hysterectomies through the National Disease Research Interchange (NDRI, Philadelphia, PA) and transported overnight in RPMI medium.

3 Active pharmaceutical ingredients (APIs) for in vitro experiments

MIV-150 was developed by Medivir AB (Sweden) and licensed to the Population Council (Kenney, Aravantinou et al. 2011). LNG was purchased from CrystalPharma (Valladolid, Spain). Positive toxicity control Gynol (4% Nonoxynol 9 was purchased from www.drugstore.com.

4 Gels

PC-1005 contains 2.8% (wt/vol) CG, 0.002% (wt/vol) MIV-150 (50 µM) and 0.3% (wt/vol) zinc acetate dihydrate (ZA) (14 mM). PC-525, a CG placebo, gel containing 2.6% (wt/vol) CG (lot 130613A525TR) was used as a control in the in vitro studies. Hydroxycellulose (HEC; universal placebo gel) was used in the Phase 1 study. All gels were manufactured and packed at the Population Council.

PC-1005 and PC-525 used to study their activity in macaque explants were lot 130605A1005TR and lot130613A525TR respectively. To determine the activity of the gels in human ectocervical explants *in vitro* (gels applied directly in the explant model) the gels belonged to lot 130605A1005TR. The PC-1005 gel that was applied to the subjects in the Phase 1 clinical trial belonged to lot 131118A1005MC.

5. Intravaginal rings (IVRs)

Core-matrix IVRs were prepared as described in (Ugaonkar, Wesenberg et al. 2015). Briefly, 20mm x 4mm IVRs consisted of an ethylene vinyl acetate 40 (EVA) matrix ring body loaded with 3 mg MIV-150/0.6 mg LNG and a solid core filled with 30 mg ZA /70 mg CG (MZCL IVR) or an EVA matrix containing 3 mg MIV-150 and 30 mg ZA/70 mg CG in the core (MZC IVR). LNG IVRs consisted of an EVA/0.6 mg LNG matrix and an empty core. A 500 µm core-side pore was drilled into the IVR to release ZA and CG. (Fig 2)

Figure 2

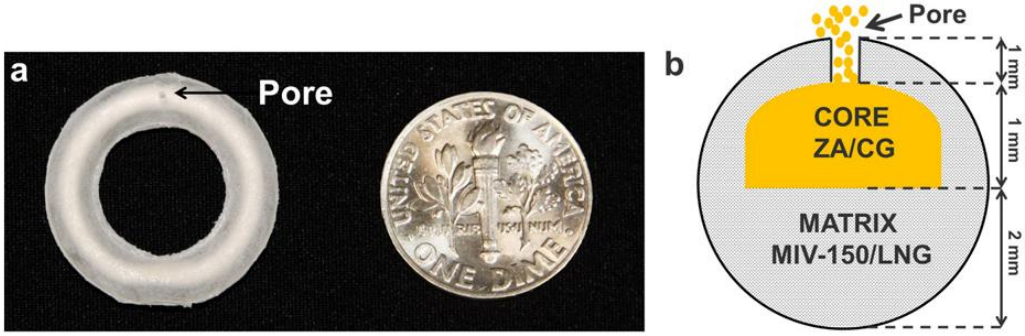


Fig. 2-Core–matrix IVR. (a) Photo of the MZCL IVR (20mm×4mm),macaque prototype, containing ZA/CG core (off-white ring) as seen through the translucent EVA-28matrix containing MIV-150 and LNG and a pore to elute hydrated ZA/CG gel (scale: US dime=17.91mmdiameter). (b) Cross sections depicting core and matrix compartments of the same IVR with a core side pore eluting ZA/CG gel.(Adapted from Ugaonkar et al. 2015)

6 Viral stocks

6.1 PC-1005 gel and VF in macaque mucosae ex vivo: SHIV-RT is a chimeric virus that expresses the HIV-1 HxB2 reverse transcriptase (Balzarini, Weeger et al. 1995), offering a convenient tool for the evaluation of reverse transcriptase inhibitors in non-human primates ex vivo and in vivo (Balzarini, De Clercq et al. 1997; Jiang, Tian et al. 2009; Jiang, Tian et al. 2009; Pal, Nuttall et al. 2009). SHIV-RT was generated from the original stock provided by Disa Böttiger (Medivir AB, Sweden) (Turville, Aravantinou et al. 2008) using PHA/IL-2-activated macaque PBMCs (Kenney, Aravantinou et al. 2011).

6.2 Tissue associated MIV-150. To prepare SHIV-RT stock for tissue PD studies, macaque peripheral blood mononuclear cells (PBMCs) were depleted of CD8+ cells and activated for 6 days in the presence of 10 nM retinoic acid (RA) (Sigma Aldrich, St. Louis, MO), 20 U/ml IL-2 (NCI BRB Preclinical Repository, Frederick, MD) and 50 ng/ml of anti-CD3 mAbs (clone OKT3; e-Bioscience, San Diego, CA) in complete RPMI 1640 (Cellgro Mediatech, Manassas, VA) containing 10% FBS (Life Technologies, Grand Island, NY), 100 U/ml penicillin - 100 µg/ml streptomycin (Cellgro Mediatech) (Arthos, Cicala et al. 2008; Nawaz, Cicala et al. 2011). Activated PBMCs were then challenged with 10^3 50% Tissue culture Infective Dose ($TCID_{50}$) of SHIV-RT per million cells. Titration of both SHIV-RT productions was performed in CEMx174 cells as in (Kenney, Aravantinou et al. 2011).

6.3 PC-1005 gel and cervicovaginal lavages(CVL) in human cervical tissues: For the generation of HIV-1_{BaL} (group M, clade B), PBMCs were isolated from normal leukocyte-enriched blood of anonymous donors (New York Blood Center, Long Island City, NY) using Ficoll-Hypaque density gradient centrifugation, depleted of $CD8^+$ cells using anti-$CD8^+$ magnetic beads (Miltenyi Biotech Inc., San Diego, CA) and activated for 6 days with 10 nM Retinoic Acid (RA) (Sigma Aldrich, St. Louis, MO), 20 U/ml IL2 (NCI BRB Preclinical Repository, Frederick, MD) and 50 ng/ml of anti-CD3 mAbs (clone OKT3; e-Bioscience, San Diego, CA) in RPMI 1640 (Cellgro Mediatech, Manassas, VA) containing 10% FBS (Life Technologies, Grand Island, NY)(Arthos, Cicala et al. 2008; Nawaz, Cicala et al. 2011). Activated cells were then challenged with 10^3 $TCID_{50}$ of HIV-1_{BaL} (ABI, Eldersburg, MD) per 10^6 cells and cultured until p24 concentration>100 ng/ml (7-8 days post challenge) in the presence of 10 nM of RA and 20 U/ml IL-2. The 50% tissue culture infectious dose (TCID50) was determined in TZM-bl cells (28). To determine whether the HIV-1_{BaL} stock harbors NNRTI drug resistant mutations (DRMs) the reverse transcriptase/RNAse genes of the *pol* region were sequenced. RNA was extracted from an aliquot of HIV-1_{BaL} with QIAamp UltraSens Virus RNA kit (Qiagen, Valencia, CA) and reverse-transcribed using SuperScript III

First-strand Supermix and random hexamer primer (Invitrogen, Carlsbad, CA) as previously described (30). PCR amplification was carried out to amplify RT-RNase H region of pol gene with primers Pol Fwd: 5' AGAGCCAACAGCCCCACCA 3' and Pol Rev: 5' CACCTGCCATCTGTTTTCCA 3'. PCR products were purified with QIAquick gel extraction kit (Qiagen) and sent for sequencing (GeneWiz, South Plainfield, NJ) with primers specific for HIV-1 RT. Gene sequences were analyzed by Lasergene 10 (DNAStar, Madison, WI). No NNRTI DRMs were detected.

6.4 HSV-2 strain G. HSV-2 was propagated in Vero cells (ATCC) as described and the viral titer in pfu/ml was obtained by plaque formation assay on monolayer cultures of Vero cells (Ashley 1995).

A single HIV-$1_{BaL,}$ SHIV-RT and HSV-2 were used for each study. Stocks were aliquoted and stored at −80°C.

7 Samples

7.1 Macaque vaginal mucosa for *in vitro* PC-1005 activity. Necropsy vaginal tissue (at least 0.5-1cm) and vaginal biopsies (n=2 at each collection time; 3x5mm each) were utilized. Tissues (biopsies, necropsy) were transported in complete L-15 medium (Ouattara, Barnable et al. 2014) to our laboratory on ice overnight and then cut into 3 mm diameter explants using skin biopsy punches (Acu-Punch, Acuderm, Fort Lauderdale, FL)(Crostarosa, Aravantinou et al. 2009; Ouattara, Barnable et al. 2014). Explants were pooled and randomly assigned treatments before viral challenge (below).

7.2 Rectal mucosa. 20 x 1.5 mm diameter biopsies procured at each collection time.

7.3 MIV-150/LNG toxicity, anti-SHIV-RT activity in vitro and ex vivo (VF PD). Necropsy vaginal tissue specimens were available from n=16 Chinese and Indian macaques. Vaginal biopsies were collected from n=11 Chinese and Indian macaques. Animal ages ranged from 5.7-22 years old and their weights ranged from 4.3-12 kg. Vaginal fluids were available from SHIV/HSV-2 Chinese and Indian macaques with MZC (n=11) and MZCL (n=9) IVRs inserted for 21 or 28d in previously completed studies (Ugaonkar, Wesenberg et al. 2015). Animal ages ranged from 5.25 - 13.15

years old and their weights ranged from 5.5 - 14 kg.

7.4 *In vivo* IVR study (API concentrations and tissue PD). Vaginal and cervical biopsies, blood samples and VF (swabs) were derived from n=10 Indian macaques. Animals were inserted MZCL (n=5) and LNG (n=5) IVRs for 24h. Animal ages ranged from 7 - 12.7 years old and their weights ranged from 6 - 12 kg.

7.5 Human ectocervical tissues (~1.5 x 1.5 cm pieces) were obtained from NDRI and transported overnight in RPMI medium.

8 Tissue processing

8.1 Macaque

8.1.1 Vaginal mucosa and VF collection. For the in vitro PC-1005 gel and IVR VF activity, tissues were transported overnight and cut into 3mm diameter explants using skin biopsy punches (Acu-Punch, Acuderm, Fort Lauderdale, FL) as described in (Barnable, Calenda et al. 2014; Barnable, Calenda et al. 2015) before viral challenge (below). Explants were pooled and randomly assigned treatments.

8.1.2 VF from macaques with MZC (n=9) and MZCL (n=11) IVRs were available from previously completed studies (Ugaonkar, Wesenberg et al. 2015) . VF were collected using Merocel® spears (Medtronic Xomed, Jacksonville FLA) at the baseline (right before IVR insertion), and at 4, 48, 72h and 9d after insertion and placed in saline and processed as in (Crostarosa, Aravantinou et al. 2009). Aliquots of VF were stored at -80°C. VF MIV-150 and CG concentrations were measured as described below.

8.1.3 In vivo IVR study (API concentrations and tissue PD). Vaginal and cervical biopsies, blood samples and VF were derived from n=10 Indian macaques. Animals were inserted MZCL (n=5) and LNG (n=5) IVRs for 24h. Animal ages ranged from 7 - 12.7 years old and their weights ranged from 6 - 12 kg. Biopsies and VF were collected at baseline and after IVR removal. Biopsies were processed for viral challenge immediately after collection at TNPRC.

8.1.4 Rectal mucosa (1.5 mm biopsies) was processed for viral challenge (below) at TNPRC immediately after collection.

8.2 Human

8.2.1 Tissues: to normalize mucosal area 3 mm explants were cut from ectovervical tissues using skin biopsy punches (Acu-Punch, Acuderm, Fort Lauderdale, FL) before challenge or

exposure to CVLs.

8.2.2 Cervicovaginal lavages: CVLs were available from 15 participants administered gels vaginally once a day for 14 days in PC-1005 Phase 1 trial or Hydroxycellulose universal placebo (HEC)(Friedland, Hoesley et al. 2016). CVLs were collected using 10ml of saline at the baseline and 4h (n=6 PC-1005 and n=2 HEC) or 24h (n=6 PC-1005 and n=1 HEC) post last gel administration.

9 P24 and p27 ELISA

HIV-1_{BaL} and SHIV-RT infection in tissue culture supernatants was measured by p24 ELISA and p27 ELISA respectively (ZeptoMetrix, Buffalo, NY)

10 SIV and HIV *gag* qRT-PCR and HSV-2 *pol* qPCR.

SHIV-RT and HIV-1_{BaL} infection were monitored directly in 5 µl of tissue culture supernatants by a one-step SIV and HIV *gag* reverse transcription quantitative PCR using KAPA SYBR FAST One-step qRT-PCR Kit (KAPA Biosystems, Wilmington, MA). Specific SIV and HIV *gag* primers (Integrated DNA Technologies, Coralville, IA) were SIV667 *gag* (5′ GGTTGCACCCCCTATGACAT 3′), SIV731 *gag* (5′ TGCATAGCCGCTTGATGGT 3′) and HIV *gag* FW (5’ GCTCGAGTGCGAAAAAACGTTC 3’) and REV (5’ TGCGGTTGATAAACGCGCAGT 3’). Results were analyzed by the standard curve method, using SIVmac1A11 and pNL (AD8) DNA respectively obtained from Dr. Paul Luciw through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. Infection supernatants were lysed/inactivated prior qRT-PCR by heating at 95°C for 5 minutes followed by a rapid cooling. Cycling conditions were: Step 1: 1× 42°C 5 min, Step 2: 1× 95°C 5 min, Step 3:40× (95°C 3 sec, 60°C 20 sec). (Goode, Truong et al. 2014; Villegas, Calenda et al. 2016; Villegas, Calenda et al. 2016). The LLOQ of the assay is 10 copies /5µl. The coefficients of inter/intra-assay variability are 1.55 and 1.32 for SIV *gag* and 2.58/ 0.6 respectively for HIV *gag* respectively.

HSV-2 infection was analyzed by HSV-2 *pol* qPCR in supernatants (5 µl per reaction). Briefly, primers (IDT, Coralville, IA) that amplify the UL30 region of the HSV-2 polymerase gene were used: FW (5’ GCTCGAGTGCGAAAAAACGTTC 3’) and REV (5’ TGCGGTTGATAAACGCGCAGT 3’). qPCR was performed using the KAPA SYBR FAST qPCR kit (Kapa Biosystems, Wilmington, MA). Cycling conditions were: 95°C 3 min, 40x (95°C 3″, 60°C 20″). The standard curve was generated using 10 fold dilutions of the plasmid TOPO UL30 (Goode, Truong et al. 2014; Villegas, Calenda et al. 2016).

In all qPCRs dissociation curves were generated to verify absence of unspecific amplification. PCRs were performed and data were analyzed using the ViiA™ 7 real time PCR system and software respectively (Applied Biosystems, Carlsbad, CA).

11 MIV-150, LNG and CG quantification

MIV-150 was measured by Liquid chromatography–mass spectrometry (LCMS/MS) in plasma with a lower limit of quantification (LLOQ) = 20 pg/ml (Kenney, Singer et al. 2012), in tissues (LLOQ = 25 pg/mg) and in CVLs (LLOQ= 100 pg/ml) (Kizima, Rodriguez et al. 2014), and by Radioimmunoassay (RIA) in VF (LLOQ = 1 ng/ml) (Kenney, Aravantinou et al. 2011). LNG was measured by RIA in serum (LLOQ=47 pg/ml) (Oregon National Primate Research Center (ETSC, Beaverton, OR). CG was measured by ELISA in VF and (LLOQ= 40 ng/ml) (Kizima, Rodriguez et al. 2014) and in CVLs by LCMS/MS (LLOQ=58 ng/ml).

12 Tissue viability by MTT

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color.

To assess tissue viability MTT was performed in triplicates after overnight (~18h) exposure to LNG, MZC or CG gels, and CVLs as previously described (Aravantinou, Singer et al. 2012; Barnable, Calenda et al. 2014; Ouattara, Barnable et al. 2014). Briefly tissues were washed and cultured in cDMEM containing 0.5mg/ml of MTT (Sigma Aldrich) for 2h at 37°C. After incubation, each explant was transferred to 1ml of methanol overnight in the dark. Then 200µl of the extractant solution (in triplicate) were measured spectrophotometrically at 570nm. The tissues were dried for at least 24h. Tissue viability was determined by normalizing the optical density at 570nm (OD570) of the formazan product by the dry weight of the explants.

13 Comparison of SHIV-RT growth kinetics using qRT-PCR and p27 ELISA

To compare SHIV-RT infection readout methods in vaginal explants, tissues were processed and challenged with SHIV-RT as in (Barnable, Calenda et al. 2014). Briefly, vaginal explants were stimulated (5 µg/ml PHA (Sigma Aldrich) and 100 U/ml IL-2 (NCI BRB Preclinical Repository, Frederick, MD) for 48h and then challenged with 10^4 $TCID_{50}$ SHIV-RT per explant for ~18h. Following washout, explants were cultured in the presence of IL-2 for 14d (Barnable, Calenda et al. 2014). Supernatants were collected on days 0, 3, 7, 11, and 14 and infections levels were analyzed by SIV gag one-step qRT-PCR and p27 ELISA. To compare SHIV-RT infection readout methods in rectal biopsies, supernatants from SHIV-RT/HSV-2 co-challenge experiments (below)

were used. Controls included 10 µM 3TC or 10 µM of 3TC/100 µg/ml Acyclovir.

14 Viral challenge of tissues to evaluate APIs activity.

14.1 SHIV-RT and HSV-2 co-challenge of vaginal and rectal mucosa and antiviral activity of PC-1005. Phytohemagglutinin / Interleukin 2 (PHA/IL-2) stimulated vaginal explants and unstimulated rectal biopsies were co-challenged with 10^4 $TCID_{50}$ SHIV-RT and 10^6 pfu HSV-2 per explant for ~18 (vaginal) or 4h (rectal). To test the antiviral activity of PC-1005, viral challenge was done in the presence 1:300 or 1:100 diluted PC-1005 (MZC) and PC-525(CG) gels vs. untreated (Medium) and 3TC/Acyclovir controls. Following washout, vaginal explants were cultured in the presence of IL-2 for 14d (Barnable, Calenda et al. 2014). Rectal biopsies (n=4) were placed on 12mm diameter Gelfoam sponges (Ethicon, Somerville, NJ) presoaked in DMEM (Cellgro Mediatech) containing 10% FBS (Gibco, Life Technologies, Grand Island, NY), 100 U/ml penicillin, 100 µg/ml streptomycin (Cellgro Mediatech), 100µM of non-essential amino acids (Irvine Scientific, Santa Ana, CA) at 37ºC, 5% CO2 for at least 30 minutes and cultured for 14d (no IL-2). Rectal biopsies were cultured in cDMEM to which 80 µg/ml gentamicin had been added (Gibco). Supernatants from both vaginal and rectal tissues were collected on days 0, 3, 7, 11, and 14 and levels of infections were analyzed by qPCR and/or ELISA.

14.2 In vitro anti-HIV-1_{BaL} and anti-HSV-2 activity of PC-1005 in human ectocervical tissues. The design of in vitro experiments testing PC-1005 activity is summarized in Fig. 3A. Ectocervical tissues were challenged with 500 $TCID_{50}$ HIV-1_{BaL} or 500 $TCID_{50}$ HIV-1_{BaL}/10^6 pfu HSV-2 per explant (three explants per condition) in the presence of 1:100 and 1:300 (184.2 ng/ml and 61.4 ng/ml of MIV-150, respectively) diluted PC-1005, PC-525, 10 µM 3TC and/or 100 µg/ml Acyclovir or culture medium. These concentrations of MIV-150 are within the range of concentrations detected in vaginal fluids 4h and 24h post last gel application in Phase 1 PC-1005 study, adjusting for dilutions during CVL collection. After overnight ~18h incubation at 37ºC and 5% CO2, tissues were washed to eliminate input virus and cultured in cDMEM for 14d. Supernatants were collected at 0 (after washes), 3, 7, 11 and 14 days of culture. Infections were monitored with HSV-2 qPCR and HIV gag qRT-PCR. Endpoint SOFT and cumulative (CUM) analyses (d3-14 as d0 represents carryover input virus) were performed (Richardson-Harman, Lackman-Smith et al. 2009; Richardson-Harman, Mauck et al. 2012; Ouattara, Barnable et al. 2014).

14.3 Anti-HIV-1_{Bal} and anti-HSV-2 activity of CVLs in human ectocervical tissues. The design of experiments testing activity of CVLs is summarized in Fig. 3B. Ectocervical explants

were incubated with diluted (1:2) CVLs in cDMEM for 4h. Following washout, tissues were challenged with 500 $TCID_{50}$ HIV-1_{BaL} or 500 $TCID_{50}$ HIV-1_{BaL}/10^6 pfu HSV-2 per explant (three explants per condition), washed, cultured and infections were monitored as above. Paired (baseline and post gel) CVLs were tested in ectocervical tissues from the same donor. Controls included tissues not exposed to CVLs (Medium) and challenged with HIV-$1_{BaL,}$ HIV-1_{BaL}/HSV-2 or challenged with HIV-1_{BaL}/HSV-2 in the presence of 3TC/Acyclovir. Eight surgical ectocervical tissues were used to test activity of 15 CVL pairs. To determine the contribution of CG to anti-HIV activity of MIV-150 in CVLs, tissues were challenged with HIV-1_{BaL} after exposure to baseline pooled CVLs spiked with the range of CG concentrations (three explants per condition) detected in the post-gel application CVLs (n=3 experiments).

14.4 MIV-150/LNG toxicity and anti-SHIV-RT activity *in vitro*. Viability of explants following ~18h incubation with MIV-150 and LNG (vs. gynol) was tested by MTT assay as described in (Aravantinou, Singer et al. 2012). To test anti-SHIV RT activity, vaginal explants were prepared from biopsy and necropsy tissues, stimulated with 5 µg/ml PHA (Sigma Aldrich) and 100 U/ml IL2 in cDMEM for 48h (Ouattara, Barnable et al. 2014). Then, tissues were challenged with 10^4 $TCID_{50}$ SHIV-RT per explant in the presence of MIV-150 (1.5 and 0.15 µM), LNG (6 and 0.6 µM) alone or in combination (three explants per condition). After ~18h tissues were washed and cultured in the presence of IL2 for 14d. Infection was monitored by one step SIV *gag* qRT-PCR (as above) on tissue culture supernatants collected at days 0 (post last wash), 3, 7, 11 and 14 (Goode, Truong et al. 2014). SOFT and cumulative (CUM) analyses (d3-14 as d0 represents carryover input virus) were performed as described in (Richardson-Harman, Lackman-Smith et al. 2009; Richardson-Harman, Mauck et al. 2012; Ouattara, Barnable et al. 2014).

Figure 3

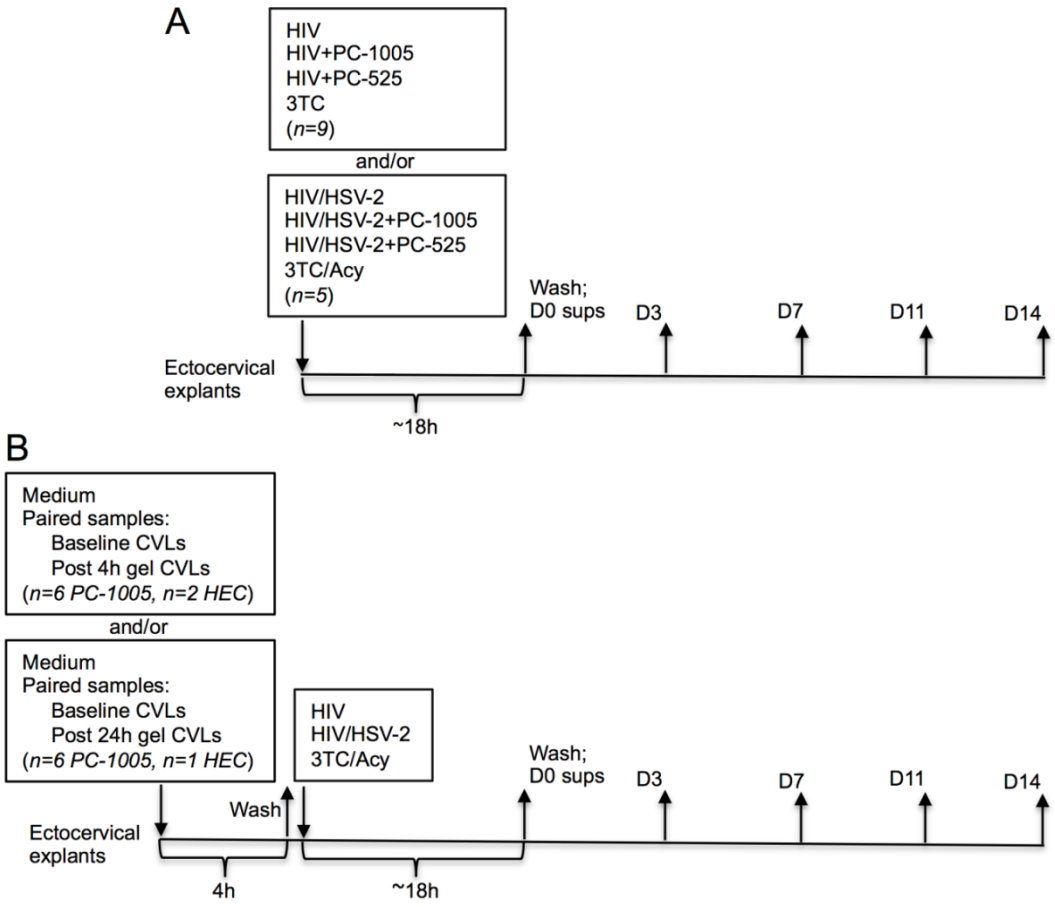


Fig. 3-Design of experiments testing activity of PC-1005 in vitro and activity of CVLs collected from subjects in PC-1005 Phase 1 trial. Two designs were chosen. (A) Ectocervical tissues were challenged HIV-1_{BaL} or HIV-1_{BaL}/HSV-2 in the presence of PC-1005 vs. controls. Following challenge, tissues were washed and cultured. Alternatively, (B) ectocervical tissues were pre-incubated with CVLs, then washed and challenged with HIV-1BaL or HIV-1BaL/HSV-2. After challenge, tissues were washed and cultured for 14d.(From (Villegas, Calenda et al. 2016)

14.5 VF PD. Vaginal explants were prepared from biopsies and necropsies as above. Anti-SHIV-RT activity of MIV-150 released in macaque VF was tested in vaginal explants as described in (Ouattara, Barnable et al. 2014). Briefly, PHA/IL2 stimulated explants were challenged with SHIV-RT as above in the presence of 1:5 diluted VF (two-three explants per condition). Then tissues were washed and cultured in cDMEM containing IL2. Infection (pooled replicates) was monitored by RETRO-TEK SIV p27 Antigen ELISA (ZeptoMetrix, Buffalo, NY). p27 SOFT and CUM endpoint analyses (d3-14) were performed as above.

15 *In vivo* IVR study (APIs concentrations and tissue PD)

MZCL (n=5) and LNG (n=5) IVRs were inserted in macaques for 24h. Vaginal fluids were collected immediately before IVR removal as above, placed in PBS/1%FBS and processed as described in (Crostarosa, Aravantinou et al. 2009) and stored at -80°C until assayed for MIV-150 and CG concentrations. Blood was collected immediately before IVR removal. Serum LNG, plasma MIV-150, VF MIV-150 and VF CG concentrations post IVR removal were measured as below.

Vaginal (n=2 per time point; 3x5mm each) and cervical (n=2 per time point; 3x4.5mm each) (Ouattara, Barnable et al. 2014) biopsies were collected 24h post IVR insertion and at the baseline (performed 5 weeks after IVR removal). One animal in the LNG group developed recto-vaginal fistula and was euthanized. Therefore, the data include n=5 and n=4 animals in MZCL and LNG groups, respectively. In 2 out of 4 animals in the LNG group, ectocervical biopsies were not available. Tissues were processed immediately for *ex vivo* challenge as described below. A portion of vaginal biopsies (5-21 mg) collected post IVR insertion was kept at -80°C until assayed for MIV-150 concentration.

Anti-SHIV-RT activity of tissue-associated MIV-150 was determined in biopsies collected 24h post MZCL and LNG IVRs insertion (vs. baseline). Biopsies were rinsed with PBS, cut into 3 mm diameter explants using skin biopsy punches as described above within an hour of collection and challenged with 10^4 $TCID_{50}$ SHIV-RT per explant (2-6 vaginal and 2-4 cervical explants) in the presence of 100 U/ml IL2. ~18h after challenge, tissues were washed and cultured in cDMEM containing IL2 for 14d (Ouattara, Barnable et al. 2014). Supernatants were collected at d0 (after washes), 3, 7, 11 and 14d of culture (Ouattara, Barnable et al. 2014). Infection was monitored by one step SIV *gag* qRT-PCR as above (Goode, Truong et al. 2014; Villegas, Calenda et al. 2016; Villegas, Calenda et al. 2016). SIV *gag* SOFT and CUM endpoint analyses (d3-14) were performed as above.

Statistical analysis

16 PC-1005 gel and CVLs activity

16.1 Comparison qPCR vs p27 ELISA. The sensitivity of paired p27 ELISA and SIV *gag* qRT-PCR results was compared using McNemar's test and inter-rater agreement (Kappa) statistics using GraphPad calculators available online (graphpad.com).

16.2 Tissue viability. A log-normal generalized linear mixed model predicted the weight-normalized OD_{570} of each tissue replicate post exposure to gels or to CVLs. A random intercept

and treatment slope were included by tissue subject ID. The significance was determined by the Type 3 *F*-test for overall treatment effect.

16.3 Analysis of gel activity against SHIV-RT and HSV-2 in macaque tissues explants was performed as described in (Richardson-Harman, Lackman-Smith et al. 2009; Richardson-Harman, Mauck et al. 2012; Barnable, Calenda et al. 2014; Ouattara, Barnable et al. 2014; Barnable, Calenda et al. 2015) using a log-normal generalized linear mixed model with SOFT or CUM as the response and gel treatment as the predictor. For the experiments using rectal biopsies, a random intercept for each animal was included. For the vaginal experiments, a random intercept was included for each biopsy. All analyses were performed with SAS V9.4, SAS/STAT V13.1 with α=0.05. Significant *p*-values of <0.05 (*), <0.01 (**), and ≤0.001 (***) are indicated.

16.4 HIV-1_{Bal} infection in a single and co-infection models. A paired *t* test on log-transformed values was used to test whether levels of infection of HIV-1_{Bal} were different between a single infection and co-infection settings.

16.5 Activity of PC-1005 and PC-525 against tissue HIV-1_{BaL} and HSV-2 infection. SOFT and CUM HIV *gag* and HSV-2 *pol* (pooled replicate values) were analyzed using log-normal generalized linear mixed models with treatment as the only predictor. A random intercept for each sample was included. Pairwise tests were performed with Tukey-adjusted *t* tests. For CUM and SOFT analyses, HIV *gag* and HSV-2 *pol* copy number of pooled values ≥ LLOQ (2000 copies/ml) were assumed log-normal. Any value <LLOQ at 3-14d of culture was set to $2000^{1/\sqrt{2}}$ =215.86, a common substitution for log-normal data. CUM from 3-14d for replicates with pooled values < LLOQ for all time points corresponds to 863.44 copies/ml.

16.6 Activity of CVLs against tissue HIV-1_{BaL} and HSV-2 infection. The PC-1005 Phase I trial was not designed for prediction. The fact that there are replicates per tissue sample in each experiment in addition to there being few independent tissue samples led to a choice between minimizing type I (probability of a false positive result) and type II (probability of false negative result) error rate. The choice to use linear mixed models was predicated as that minimizing the type I error is paramount, by accounting for variability within tissue sample replicates and the variability between different tissue samples. It is important to note, moreover, the reduction of the type II error rate will be minimal at best, as the aforementioned trial was not powered to evaluate any hypotheses. Mixed models are the only method that does not treat tissue replicates of each experiment as independent measurement, which indirectly leads to a false positive result by

artificially suppressing the overall model variance. SOFT and CUM HIV *gag* and HSV-2 *pol* (individual replicate values) were analyzed using log-normal generalized linear mixed models with treatment (PC-1005 vs. HEC placebo), time point (pre-exposure, 4h post-exposure, or 24h post-exposure to gels), and the treatment–time point interaction as predictors. A random intercept and time-point slope by subject was included with either a Cholesky-root-parameterized unstructured covariance matrix or, if that failed to converge, a heterogeneous compound symmetric or then a compound symmetric structure. Tests of effects were done with *F* tests. Pairwise tests of least-squares means were performed with *t* tests adjusted by stepdown simulation for multiple comparisons. The association of MIV-150 and CG concentrations with anti-HIV and anti-HSV-2 activity in CVLs, respectively, was analyzed using log-normal generalized linear mixed models on the individual replicate values to predict the SOFT and CUM (individual replicate values). The analysis was done including baseline and placebo gel data. The MIV-150 or CG concentration was the only predictor. MIV-150 and CG values < LLOQ (100 pg/ml for MIV-150 and 58 ng/ml for CG) were set to $100^{1/\sqrt{2}}$= 0.025 ng/ml and $58^{1/\sqrt{2}}$=0.017 µg/ml, respectively. A random intercept was included by subject.

17 IVR studies

17.1 MIV-150/LNG activity against tissue SHIV-RT infection *in vitro*. SOFT and CUM p27 values (pooled replicate values) were analyzed using log-normal generalized linear mixed models with treatment as the only predictor. A random intercept for each animal was included. Pairwise tests were performed with Tukey-adjusted *t* tests.

17.2 VF PD. Anti-SHIV-RT activity of MIV-150 released in VF (vs. baseline VF) was analyzed using a log-normal generalized linear mixed model to predict the SOFT and CUM p27 values (pooled replicate values). The treatment (MZC or MZCL IVRs) and the log-transformed MIV-150 concentrations were the predictors. Both the biopsy animal ID and the VF animal ID were included as random effects.

17.3 Tissue PD. For CUM and SOFT analyses, SIV *gag* copy number of individual replicate values ≥ LLOQ were assumed log-normal. Any value <LLOQ (2000 copies/ml) at 3-14d of culture was set to $2000^{1/\sqrt{2}}$ =215.86, a common substitution for log-normal data. CUM from 3-14d for replicates below LLOQ corresponds to 863.44 copies/ml. Comparisons of CUM and SOFT S*IV gag* copies endpoints were performed using a log-normal generalized linear mixed model. The treatment (MZCL vs. LNG IVRs), biopsy time (baseline vs. post IVR exposure) and their interactions were used a predictors. A random intercept and time were added as random effects

within an animal ID. Overall significance was determined by the Type 3 *F*-test, and pairwise comparisons were made with Tukey-adjusted *t* tests. To analyze if inhibition of SHIV-RT infection in the tissue is dependent on the concentration of MIV-150 in VF, plasma, or vaginal tissue, these concentrations were used separately as predictors of SOFT and CUM results in log-normal generalized linear mixed models. All models included a random intercept for each animal ID.

IV. RESULTS

PC-1005 activity in macaque vaginal and rectal tissues.

18 SIV *gag* qRT-PCR and p27 ELISA demonstrate similar SHIV-RT growth kinetics in explants with qRT-PCR being a more sensitive infection readout. To determine the feasibility of one-step SIV *gag* qRT-PCR for analysis of SHIV-RT infection in vaginal and rectal tissue cultures, a comparison with p27 ELISA was carried out. Supernatants from vaginal (n=7 experiments) and rectal (n=8 experiments) tissues challenged with SHIV-RT (vaginal) or SHIV-RT and HSV-2 (rectal) were analyzed by both ELISA and qRT-PCR at 5 time points post challenge (D0, 3, 7, 11 and 14). 3TC and 3TC/Acyclovir controls were available in n=2 vaginal tissue experiments and in all n=8 rectal tissue experiments, respectively. The results show similar SHIV-RT growth kinetics by both methods (Fig. 4). A total of n=45 (vaginal) and n=80 (rectal) data points was collected. In vaginal tissue culture supernatants, 8/45 samples had a positive readout (values ≥ LLOQ) by ELISA, while 42/45 samples had a positive readout by qRT-PCR. In rectal tissue supernatants, positive readout was obtained in 28/80 samples by ELISA, and in 65/80 by qRT-PCR. McNemar's test for matched pairs showed higher sensitivity of qRT-PCR vs. ELISA ($p < 0.0001$, both vaginal and rectal tissue experiments). Examining statistical agreement by Kappa analysis we found a Kappa coefficient of 0.03 for vaginal supernatants and 0.22 for rectal, indicating poor and fair strength of agreement, respectively. These results further emphasize higher sensitivity of qRT-PCR vs. ELISA.

Figure 4

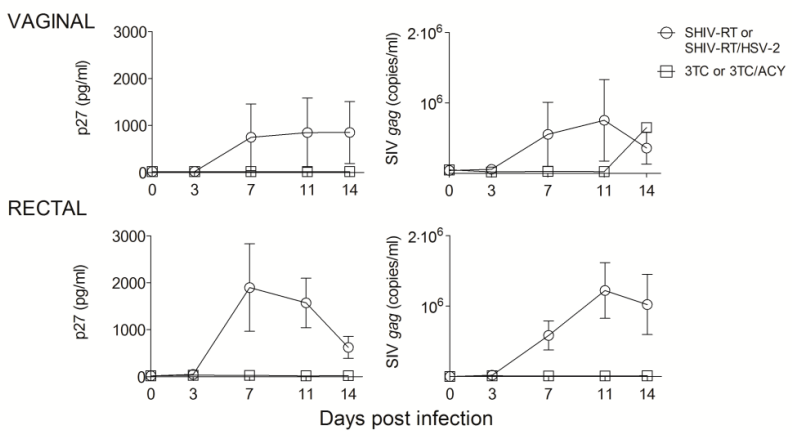


Fig 4- SIV *gag* qRT-PCR and p27 ELISA demonstrate similar SHIV-RT growth kinetic in explants. PHA/IL-2 stimulated macaque vaginal explants were challenged with 10^4 $TCID_{50}$ SHIV-RT ~18h. After washout, tissues were cultured (three explants per condition) for 14d. A summary of n=7 SHIV-RT challenge experiments (Mean±SEM) is shown. 3TC controls were included in 2 of 7 experiments. Rectal explants were challenged with 10^4 $TCID_{50}$ SHIV-RT and 10^6 pfu HSV-2 vs. 3TC/Acyclovir control for 4h. After washout, tissues were cultured (4 biopsies/well; single well per condition) for 14d. A summary of n=8 experiments (Mean±SEM) is shown**.** SHIV-RT infection kinetics were followed by one-step SIV *gag* qRT-PCR and p27 ELISA(From(Calenda, Villegas et al. 2016)

19 PC-1005 (MZC) protects macaque vaginal mucosa against SHIV-RT and HSV-2 co-infection. Stimulated vaginal explants were co-challenged with SHIV-RT (10^4 $TCID_{50}$ per explant) based on our published data (Aravantinou, Singer et al. 2012; Barnable, Calenda et al. 2014; Ouattara, Barnable et al. 2014) and with HSV-2 (10^6 pfu per explant) based on titration experiments

demonstrating robust infection with this challenge dose (not shown). Controls included co-challenged tissues cultured in the presence of 3TC and Acyclovir. Fig. 5A provides representative examples of SHIV-RT and HSV-2 growth curves. Reproducible tissue infection was achieved with both viruses (Fig. 5B). In this system, no enhancement of SHIV-RT infection was detected in the presence of HSV-2 compared to SHIV-RT alone.

We previously demonstrated that PC-1005 (MZC) gel is active against single SHIV-RT infection in PHA/IL-2 stimulated vaginal explants (Barnable, Calenda et al. 2014). In this study we aimed to determine whether PC-1005(MZC) is active against SHIV and HSV-2 in a high-dose SHIV-RT/HSV-2 co-challenge model. Stimulated tissues were exposed to SHIV-RT and HSV-2 in the presence of non-toxic (Fig. 6A) 1:100 or 1:300 diluted PC-1005 or PC-525 (placebo) gels. To allow a direct comparison with our previous work on MZC in the single infection model (Barnable, Calenda et al. 2014), p27 ELISA was used as a readout. PC-1005 (1:100 and 1:300 dilutions) strongly inhibited SHIV-RT infection relative to untreated (Medium) and CG controls (SOFT/CUM, 90-99% inhibition, p< 0.0001/0.05) (Fig. 6B). MZC and CG at 1:100 dilution (SOFT/CUM, 99% inhibition, p<0.0001) and CG at 1:300 dilution (SOFT/CUM, >90% inhibition, p< 0.05) inhibited HSV-2 vs. untreated control, pointing to CG-mediated activity of MZC against HSV-2.

Figure 5

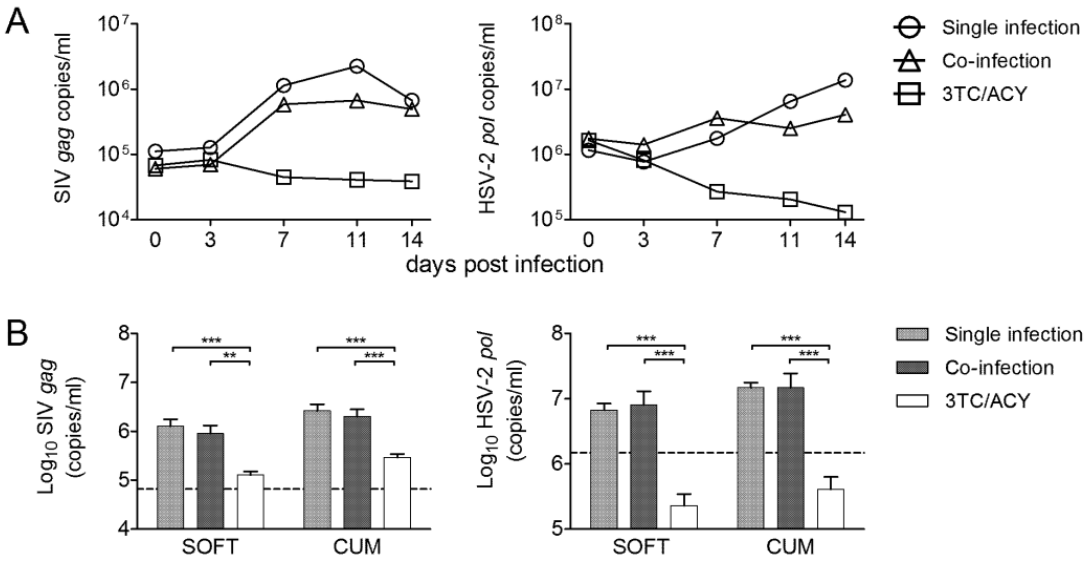


Fig 5- Macaque vaginal tissue is susceptible to SHIV-RT/HSV-2 infections after co-challenge. PHA/IL-2 stimulated macaque vaginal explants were challenged with 10^4 $TCID_{50}$ SHIV-RT and/or 10^4 $TCID_{50}$ HSV-2 (three explants per condition) vs. 3TC/Acy controls for ~18h. After washout, tissues were cultured for 14d in the presence of IL-2. (A) Representative examples of SHIV-RT and HSV-2 growth after single challenge or co-challenge are shown. Infections were monitored by one-step SIV *gag* qRT-PCR and HSV-2 *pol* qPCR. (B) Summaries of 7 experiments (SOFT and CUM analyses of SHIV-RT and HSV-2 infection) are shown. Dotted lines represent input virus (Mean) post washout at D0 (From (Calenda, Villegas et al. 2016)

C-1005 protects rectal mucosa against SHIV-RT infection

Rectal biopsies were co-challenged with 10^4 $TCID_{50}$ SHIV-RT and HSV-2 10^3-10^6 pfu per biopsy vs. 3TC/Acyclovir controls. The SHIV-RT challenge dose was chosen based on studies in vaginal tissues (above) and resulted in reproducible SHIV-RT infection (Fig. 5). In contrast, no productive HSV-2 infection was detected in rectal mucosa as similar HSV *pol* copy numbers were detected in cultures with and without Acyclovir. A differential with Acyclovir control was observed only 1 out of 10 experiments using 10^6 pfu challenge dose (not shown). We chose to test gel activity in the SHIV-RT (10^4 $TCID_{50}$) and HSV-2 (10^6 pfu) co-challenge settings to mimic possible real life HIV-1/HSV-2 co-exposure scenario.

Tissues were challenged in the presence of the non-toxic (Fig. 7A) 1:100 dilution of PC-1005 for 4h vs. untreated (Medium), PC-525, 3TC/Acyclovir controls. PC-1005 afforded significant protection against SHIV-RT relative to untreated and PC-525(CG) controls (SOFT/CUM, 92-98% inhibition, $p<0.0001$) (Fig. 7B). Similar anti-SHIV-RT activity (SOFT/CUM, 97-98% inhibition, $p<0.0001$) was detected by analysis of the same data set by qRT-PCR (not shown). As expected, HSV-2 failed to infect the rectal tissues (not shown).

Figure 6

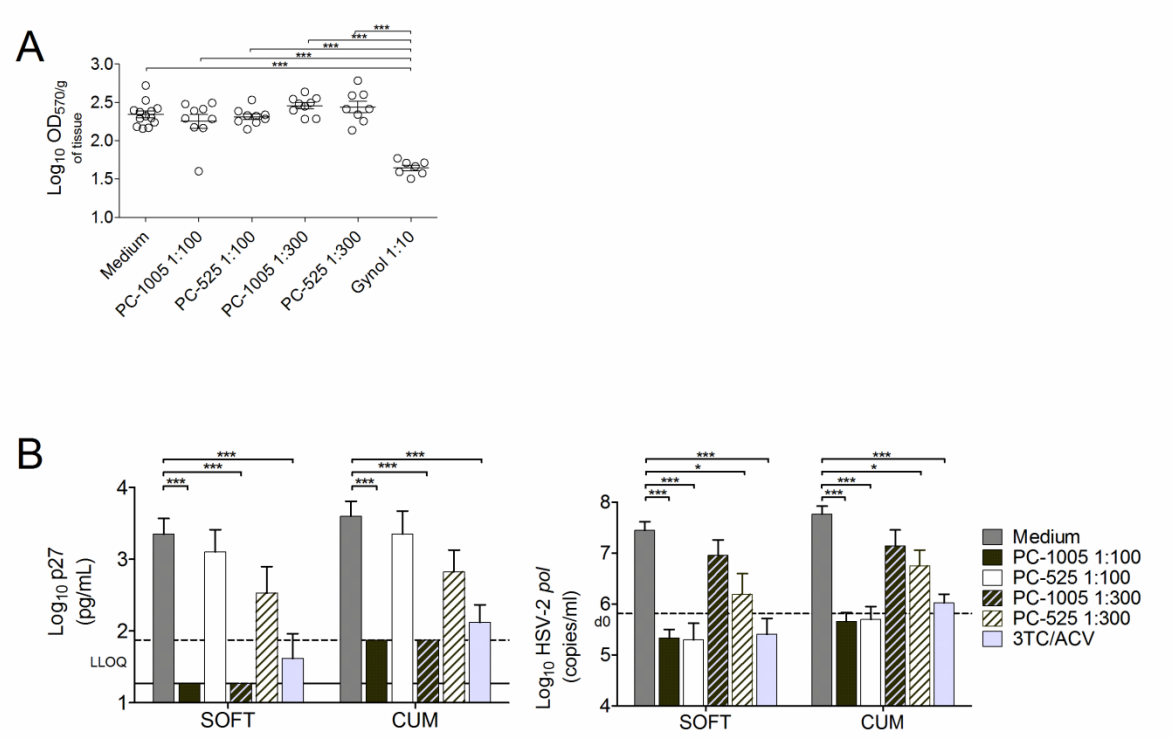


Fig 6- PC-1005 under non-toxic dilutions inhibits SHIV-RT/HSV-2 co-infection in macaque vaginal explants. (A) MTT assay was performed on tissues exposed to 1:100 and 1:300 diluted PC-1005 and PC-525 (two-three explants per condition) for ~18h. 1:10 diluted gynol served as a toxicity control. Each symbol indicates a donor and the Mean±SEM of the Log_{10} (OD_{570}/g) for each condition is shown. (B) PHA/IL-2 stimulated explants were challenged with 10^4 $TCID_{50}$ SHIV-RT and 10^6 pfu HSV-2 in the presence of 1:100 and 1:300 diluted gels (three explants per condition) vs. untreated (Medium) and 3TC/Acy controls for ~18h. Then the tissues were washed and cultured for 14d in the presence of IL2. Infections were monitored at 0, 3, 7, 11, 14d of culture by p27 ELISA and HSV-2 *pol* qPCR. Summary of 5-10 experiments (Mean±SEM of SOFT and CUM analyses) is shown. Dotted lines represent input virus (Mean) post washout at D0 (Adapted from (Calenda, Villegas et al. 2016)

es

Figure 7

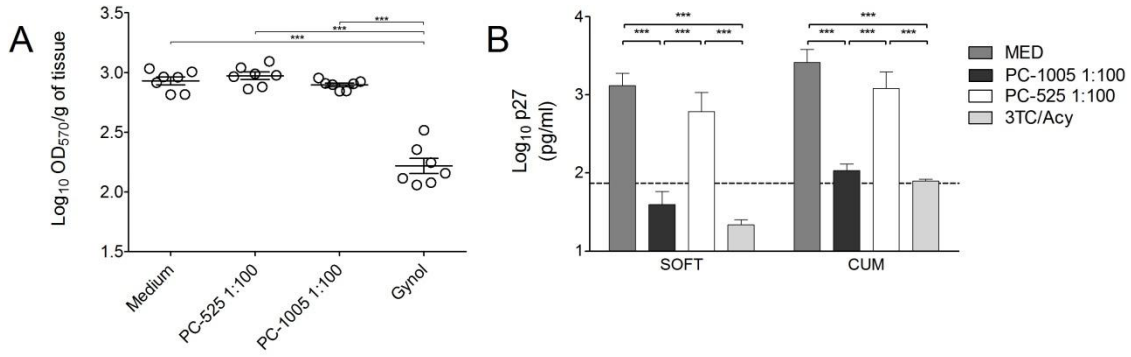


Fig 7- PC-1005 under non-toxic dilutions inhibits SHIV-RT infection in SHIV-RT/HSV-2 co-challenged macaque rectal explants. (A) MTT assay was performed on tissues exposed to 1:100 diluted PC-1005 and PC-525 (three explants per condition) for ~18h. 1:10 diluted gynol served as a toxicity control. Each symbol indicates a donor and the Mean±SEM of the $log_{10}(OD_{570}/g)$ for each condition is shown (B). Explants were challenged with 10^4 $TCID_{50}$ SHIV-RT and 10^6 pfu HSV-2 immersed in medium containing 1:100 diluted gels (three explants per condition) vs. untreated (Medium) and 3TC/Acy controls for 4h. Then the tissues were washed, transferred to Gelfoam sponges and cultured for 14d. SHIV-RT infection was measured at 0, 3, 7, 11, 14d of culture by p 27 ELISA. Summary of 8 experiments (Mean±SEM of SOFT and CUM analyses) is shown. Dotted lines represent input virus (Mean) post washout at D0. (Adapted from Calenda, Villegas et al)

PC-1005 and CVLs in human explants

21 PC-1005 inhibits HIV-1_{BaL} and HSV-2 in ectocervical tissue

Optimal HIV-1 and HIV-1/HSV-2 infection conditions were first established. Tissues were challenged with titrated doses of HIV-1_{BaL} (~50-2000 $TCID_{50}$ per explant). After virus washout, explants were cultured for 14d and infection monitored by p24 ELISA. 500 $TCID_{50}$ was selected as a middle challenge dose resulting in productive infection (Fig. 8A). Next we co-challenged tissues with 500 $TCID_{50}$ of HIV-1_{BaL} and different doses of HSV-2 (10^4-10^6 pfu per explant). Infection was monitored with HIV-1 *gag* qRT-PCR and HSV-2 *pol* qPCR. Based on reproducible infection, 10^6 pfu HSV-2 challenge dose was selected (Fig. 8B). Challenge with HIV-1_{BaL} led to robust and reproducible infection in all experiments (10 out of 10; data not shown). Seven of these experiments included the HIV-1_{BaL}/HSV-2 co-challenge condition, which resulted in HIV-1_{BaL} infection in 4 out of 7 experiments and HSV-2 infection in all experiments (Fig. 9A, B; data from the 4 successful co-infection data sets). HIV-1_{BaL} infection level in the co-challenge model was ~10 fold higher vs. single HIV-1_{BaL} infection model (SOFT/CUM; $p<0.05$; Fig. 9A) and HSV-2 infection was robust (Fig. 9B). (Villegas, Calenda et al. 2016)

To determine anti-HIV activity of PC-1005, ectocervical explants were challenged with 500 $TCID_{50}$ HIV-1_{BaL} or 500 $TCID_{50}$ HIV-1_{BaL} and 10^6 pfu HSV-2 in the presence of PC-1005 (vs. untreated, PC-525 and 3TC/Acyclovir controls). PC-1005 at non-toxic 1:100 and 1:300 dilutions inhibited >98% of HIV-1_{BaL} infection relative to untreated control (SOFT/CUM $p<0.0001$ both dilutions; Fig. 9C). Similarly, PC-1005 suppressed HIV-1_{BaL} infection (>99% inhibition) at both 1:100 and 1:300

dilutions in the more stringent co-infection model (SOFT/CUM p<0.001 and <0.01, respectively; Fig. 9D). PC-525 at the 1:100 dilution (300 µg/ml of CG) exerted a barrier effect against HIV-1_{BaL} in the single challenge model; however, this effect was not significant at 1:300 dilution (100 µg/ml of CG) of the gel (Fig. 9C). No barrier effect of PC-525 was detected in the co-challenge model (Fig. 9D). PC-1005 and PC-525 significantly (>99%) inhibited HSV-2 infection at 1:100 (but not at 1:300) dilution of the gels (SOFT/CUM p<0.05/0.01 and p<0.01/0.001, respectively) (Fig. 9E). (Villegas, Calenda et al. 2016).

Figure 8

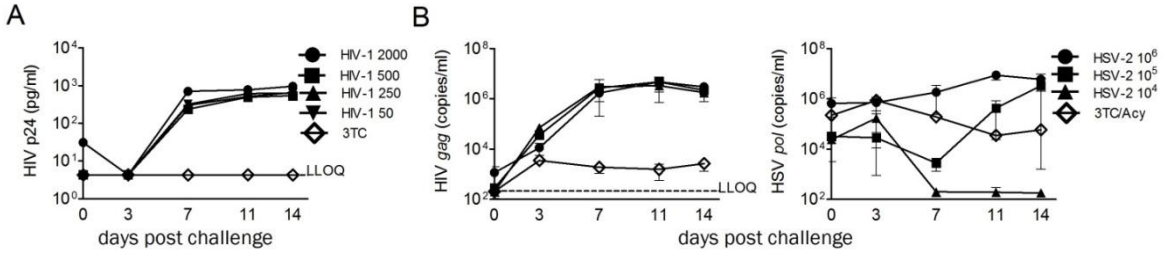


Fig. 8- Single HIV-1_{BaL} infection and HIV-1_{BaL}/HSV-2 co-infection of ectocervical tissue. Ectocervical tissues were challenged with (A) 50-2000 $TCID_{50}$ HIV-1_{BaL} or (B) co-challenged with 500 $TCID_{50}$ HIV-1_{BaL} and 10^4 - 10^6 pfu HSV-2 per explant (three explants/condition) vs. 3TC or 3TC/Acyclovir (Acy) controls for ~18h. After washout, tissues were cultured for 14d and infections were analyzed in tissue culture supernatants by p24 ELISA, HIV qRT-PCR and HSV-2 qPCR. Shown are (A) HIV p24 (pg/ml) (Mean of replicates; n=1 experiment) and (B) HIV *gag* and HSV-2 *pol* copy numbers (Mean±SEM, n=3 experiments). (Villegas, Calenda et al. 2016)

Figure 9

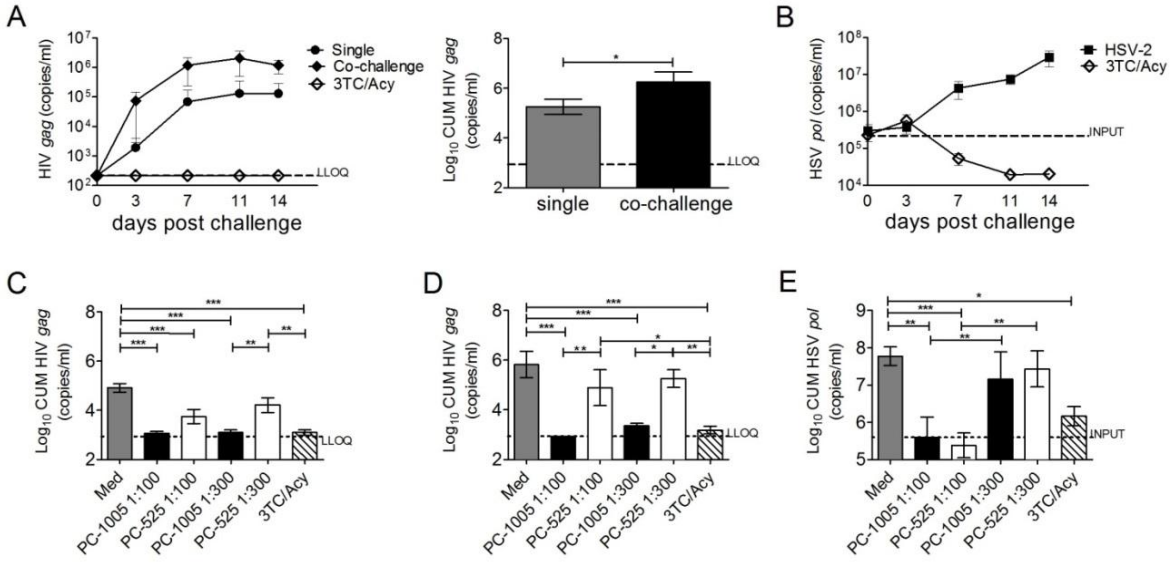


Fig. 9- Co-infection with HSV-2 enhances tissue HIV-1_{BaL} infection. PC-1005 inhibits HIV-1_{BaL} and HSV-2 infection. Ectocervical tissues were challenged with 500 $TCID_{50}$ HIV-1_{BaL} or co-challenged with HIV-1_{BaL} and 10^6 pfu HSV-2 per explant as in Fig. 8. Following washout tissues were cultured for 14d. Shown are (A) HIV *gag* copy numbers and Log_{10}-transformed CUM analysis of HIV *gag* copies numbers in paired single challenge and co-challenge experiments (Mean±SEM, n=4) and (B) HSV-2 *pol* copy numbers (Mean±SEM, n=4). Explants were challenged as above in the presence of diluted PC-1005 (vs. untreated control (Med), PC-525 and 3TC/Acy controls). Following washout tissues were cultured and infections were analyzed as in Fig. 8. Shown are Log_{10}-transformed CUM analyses of HIV *gag* copies in (C) single challenge (n=9), and (D) co-challenge (n=5) experiments (Mean±SEM) and (E) Log_{10}-transformed CUM analyses of HSV-2 *pol* copies number in the co-challenge (n=5) experiments (Mean±SEM). The LLOQ for CUM HIV *gag* and input HSV-2 *pol* copy numbers (Mean; post washout after challenge) are indicated. Significant p-values of <0.05 (*), <0.01 (**), <0.001 (***) are indicated.(Villegas, Calenda , 2016)

22 CVLs from subjects vaginally administered PC-1005 inhibit HIV-1_{BaL} and HSV-2 infection in ectocervical tissue. We aimed to test if PC-1005 administered *in vivo* is active against HIV-1_{BaL} and HSV-2 infection in mucosal cells in the presence of CVLs collected 4h or 24h post last PC-1005 gel application (vs. baseline and placebo group) in the Phase 1 PC-1005 trial(Friedland, Hoesley et al. 2016). When tissues were challenged with HIV-1_{BaL} in the presence of baseline CVLs (1:2 dilution in culture medium), the infection was inhibited (data not shown). Therefore, in CVL activity testing experiments, ectocervical tissues were pre-incubated with a non-toxic dilution of CVLs (1:2 dilution in culture medium; Fig. 10) for 4h, washed and then challenged with HIV-1_{BaL} or co-challenged with HIV-1_{BaL} and HSV-2. No impact on HIV-1_{BaL} and HSV-2 infection levels was observed under these testing conditions. In 2 out 15 co-challenge experiments no HIV infection after pre-incubation with baseline CVLs was observed. These experiments were excluded from the analysis. Comparisons of treatment (PC-1005 vs. HEC placebo gel) lacked power due to low number of placebo samples (n=2 and n=1 in 4h and 24h groups, respectively) and are not reported. As expected, higher concentrations of MIV-150 and CG were detected in CVLs obtained 4h post last gel administration vs. 24h (Fig. 11, solid circles vs. open squares). MIV-150 concentrations in CVLs inversely correlated with HIV-1_{BaL} infection in the tissues in the single challenge (SOFT/CUM, $p<0.0001$ for both) and co-challenge model (SOFT/CUM, $p< 0.01/0.05$) (Fig. 11A, B). With every 10 ng/ml increase of MIV-150, HIV-1_{BaL} infection decreased by >60% and >30% (both SOFT/CUM) in single and co-infection models, respectively. Infection inhibition was significant in the 4h (but not in 24h) post gel group vs. baseline (SOFT/CUM, $p< 0.05/0.01$) in the single challenge model. This was not the case in the co-challenge model. CG concentrations in CVLs inversely correlated with HSV-2 infection (SOFT/CUM, $p<0.01$ for both), resulting in ~30% decrease of HSV-2 infection (SOFT/CUM) per 100 µg/ml increase of CG (Fig. 11C). No significant differences between 4h and 24h groups vs. respective baselines were detected.

Figure 10

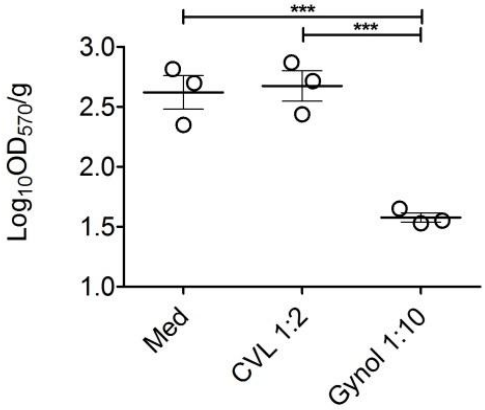


Fig. 10- Exposure to CVLs does not decrease tissue viability. Viability of ectocervical tissue after immersion in 1:2 diluted CVLs for ~18h was tested by MTT assay. OD_{570} of the formazan product was measured (three explants per condition) and normalized by the dry weight of the explants. Each symbol indicates an individual donor and the Mean±SEM of the Log_{10} OD_{570}/g of tissue for each condition is shown. A 1:10 dilution of Gynol was used as a positive toxicity control. Significant p-values of <0.001 (***) are indicated (Villegas, Calenda, 2016)

Figure 11

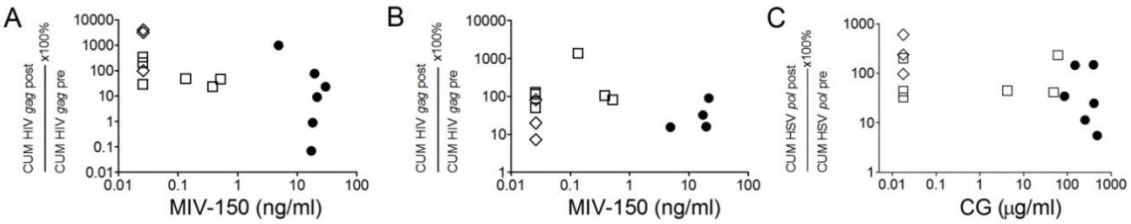


Fig. 11- MIV-150 and CG in CVLs inhibit HIV and HSV-2 infection in the ectocervical tissue in a dose-dependent manner. Tissues were incubated with paired 1:2 dilutions of CVLs (baseline and post gel) for 4 h, washed and then challenged with 500 $TCID_{50}$ HIV-1_{BaL} or 500 $TCID_{50}$ HIV-1_{BaL} and 10^6 pfu HSV-2 per explant (three explants per condition) for ~18h. Controls included tissues not exposed to CVLs (Medium) and challenged with HIV-1_{BaL}, HIV-1_{BaL}/HSV-2 or challenged with HIV-1_{BaL}/HSV-2 the presence of 3TC/Acyclovir. Following washout, tissues were cultured and infections analyzed as in Fig. 8. Changes in CUM values after exposure to post gel CVLs ("post") vs. baseline CVLs ("pre") relative to MIV-150 (A, single challenge model; B, co-challenge model) and CG (C) concentrations in diluted CVLs are shown (4h PC-1005 CVLs = solid circles, 24h PC-1005 CVLs = squares, placebo CVLs = diamonds). (Villegas, Calenda et al., 2016)

23 LNG does not alter MIV-150 activity against *ex vivo* SHIV-RT challenge in macaque vaginal explants. To explore if LNG interferes with the antiviral activity of MIV-150, PHA/IL2 stimulated vaginal explants were challenged with 10^4 $TCID_{50}$ of SHIV-RT in the presence of MIV-150 ± LNG overnight (~18h). The lowest dose of 0.15 μM (50 ng/ml) MIV-150 reflects the approximate plateau level detected in VF from MZC/MZCL IVR-treated animals (Ugaonkar, Wesenberg et al.

2015). LNG doses of 0.6-6 μM (200-2000 ng/ml) correspond to *in vitro* daily release of LNG from MZCL IVRs (Ugaonkar, Wesenberg et al. 2015). Overnight incubation with MIV-150 (1.6 μM) and LNG (up to 500 μM) did not decrease explants viability as measured by MTT assay (Fig. 12A). LNG at 0.6 μM and 6 μM did not change tissue infection level (Fig. 12B). We previously demonstrated potent activity of unformulated MIV-150 at 0.16 μM (the lowest concentration tested) against SHIV-RT in macaque vaginal explants (Barnable, Calenda et al. 2014). 0.15 - 1.5 μM MIV-150 inhibited SHIV-RT infection (vs. untreated control) and LNG doses up to 6 μM did not change activity of MIV-150 (SOFT/CUM, p>0.05). No significant differences were observed between MIV-150 - LNG and MIV-150 + LNG conditions (Fig. 12B)

Figure 12

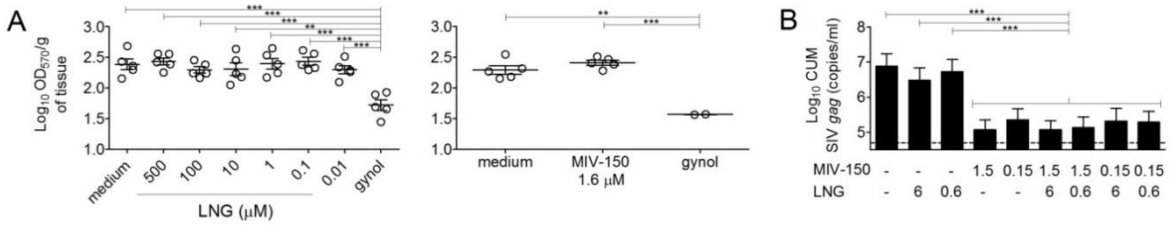


Fig. 12 - LNG does not affect the activity of MIV-150 (non-toxic concentrations) in macaque vaginal mucosa. (**A**) Macaque vaginal explants were immersed in culture media containing LNG or MIV-150 (vs. 1:10 diluted gynol) for ~18h. Tissue viability was determined using MTT assay (OD_{570} of the formazan product was measured in triplicate and normalized to the dry weight of the explants). Each symbol indicates an individual donor and the Mean±SEM of the Log_{10} OD_{570}/g of tissue for each condition is shown. (**B**) PHA/IL2 stimulated explants were challenged with SHIV-RT (10^4 $TCID_{50}$/explant; triplicates) in the presence of 1.5 or 0.15 μM MIV-150 and/or 6 or 0.6 μM LNG (vs. untreated control). 18h later, tissues were washed and cultured for 14d with IL2 and infection was monitored by SIV *gag* qRT-PCR in tissue supernatants. Summaries of Log_{10} CUM analyses (d3-14 of culture) of 7 experiments (Mean±SEM) are shown. Input SIV *gag* copy numbers (Mean; post washout after challenge) are shown as dotted lines. Significant *p*-values of <0.0001(***) and <0.001(**) are indicated. (Villegas, Calenda et al., 2016)

24 MIV-150 in VF collected from animals administered MZC and MZCL IVRs inhibits *ex vivo* SHIV-RT infection of macaque vaginal tissue in a dose-dependent manner. Anti-SHIV-RT activity of VF containing *in vivo* released MIV-150 from MZCL and MZC IVRs was tested *ex vivo*. PHA/IL-2 stimulated vaginal explants were challenged with 10^4 $TCID_{50}$ of SHIV-RT in the presence of 1:5 diluted VF collected before and 4, 48, 72h and 9d after IVR insertion. We previously demonstrated that VF do not affect the viability of macaque vaginal explants under these experimental conditions (Ouattara, Barnable et al. 2014). The 1:5 diluted VF contained 2.5-490 nM of MIV-150 (MZC and MZCL IVR groups combined). MIV-150 in VF from MZC and MZCL IVRs provided dose-dependent inhibition of SHIV-RT in vaginal tissue (combined IVR groups; SOFT/CUM p=0.02/0.01) (Fig. 13). Each time the MIV-150 concentration doubled, SOFT/CUM decreased by 39%/43%. There was not enough power to detect an association between MIV-150 in VF and protection analyzing one IVR group at a time, except for MZC IVR CUM analysis

(CUM p=0.01 for MZC IVR group; p>0.05 for MZCL IVR group). No significant difference in VF activity between the two IVRs was found for CUM, while VF from MZCL IVR-carrying animals was significantly more effective than VF from MZC IVR animals for SOFT (p=0.04). Therefore, LNG does not decrease the activity of MIV-150 in our models. Much like we observed with the MIV-150 IVR, where ≥19 nM MIV-150 in the VF strongly inhibited infection (Ouattara, Barnable et al. 2014), >20 nM MIV-150 in the VF from animals inserted MZCL IVRs strongly reduced infection.

Figure 13

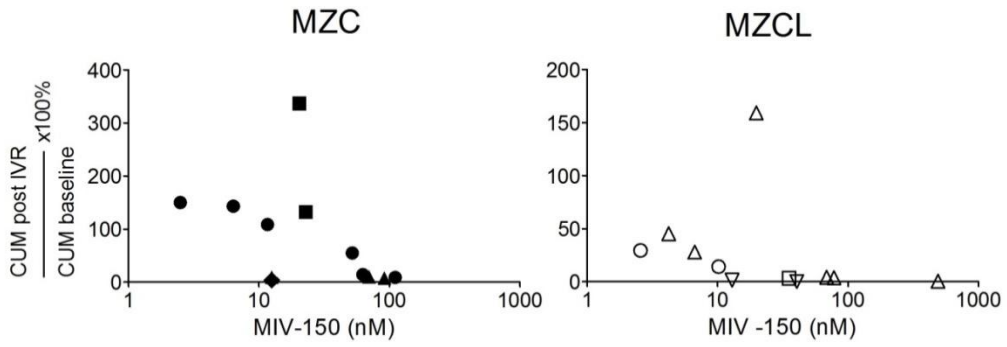
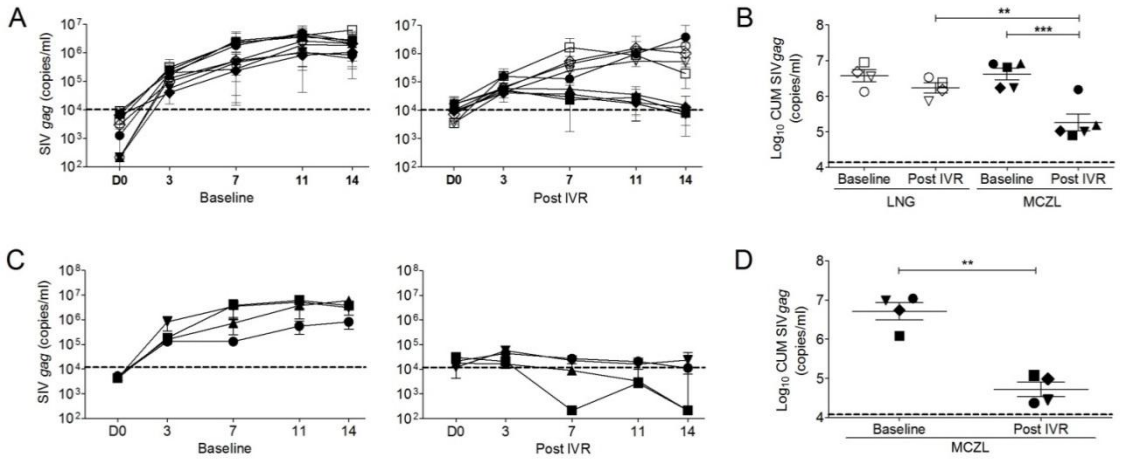


Fig. 13 - MIV-150 in VF inhibits SHIV-RT infection in vaginal mucosa in a dose-dependent manner. PHA/IL2-stimulated explants from untreated animals were challenged with 10^4 $TCID_{50}$ SHIV-RT/explant (duplicates/triplicates) in the presence of 1:5 diluted VF collected at the baseline and after MZC or MZCL IVR insertion at 4h, 48h, 72h and 9d (post IVR). The concentrations of MIV-150 correspond to 1:5 diluted VF. The explants were cultured for 14d in the presence of IL2. Infection was monitored by p27 ELISA (d0-14 of culture). The activity of each baseline IVR and post IVR VF pair was tested in explants from different donors represented by different symbols. Changes in CUM analyses (d3-14 of culture) in the presence of post IVR VF are shown relative to MIV-150 concentrations in diluted VF (Villegas, Calenda et al., 2016)

25 Tissue-associated MIV-150 inhibits *ex vivo* SHIV-RT infection. Activity of tissue-associated MIV-150 was determined in vaginal and cervical biopsies 24h post IVR insertion, at the peak of MIV-150 detected in VF, as shown in the earlier study using identical MZCL IVR (Ugaonkar, Wesenberg et al. 2015). Non-stimulated vaginal (n=2-6) and cervical (n=2-4) explants were processed for viral challenge within one hour of collection. Baseline vaginal tissue SHIV-RT infection levels were not different between IVR groups (Fig. 14 A, B). Tissue infection post LNG IVR exposure was comparable to infection at the corresponding baseline and to the MZCL group baseline (Fig. 14 A, B). In contrast, infection of vaginal tissues post MZCL IVR exposure was significantly reduced relative to the respective baseline (96% inhibition, SOFT/CUM both p< 0.0001) and the post LNG IVR group (~ 90% inhibition, SOFT/CUM; p= 0.0002/0.0007) (Fig.14 A, B). Infection of cervical tissue in the post MZCL IVR group was significantly inhibited relative to baseline (99% inhibition; SOFT/CUM p= 0.01/0.01) (Fig. 14 C, D). An insufficient number of ectocervical samples in the LNG IVR group precluded statistical analysis.

26 APIs concentrations/PD assessment. MIV-150 (VF, vaginal tissues and plasma) and LNG (serum) were measured 24h post MZCL IVR insertion (Table 1). Cervical tissues were not available for assessment of MIV-150 concentrations. The levels of MIV-150 and LNG in blood, and MIV-150 in VF were comparable to those seen in earlier pharmacokinetics (PK) studies (Ugaonkar, Wesenberg et al. 2015). As expected based on previous data (Ugaonkar, Wesenberg et al. 2015), CG was not detected in VF at this time. An average of 20.9 ng/mg of MIV-150 was detected in vaginal tissue (Table 1). We did not observe dose-dependent inhibition of SHIV-RT infection in vaginal tissue. MIV-150 concentration in VF did not predict anti-SHIV-RT activity in the vaginal and cervical tissues. Also, plasma MIV-150 concentrations did not predict anti-SHIV-RT activity in cervical tissues.(Villegas, Calenda et al. 2016)

Figure 14



Tissue-associated MIV-150 ***ex vivo*** **SHIV-RT infection of vaginal and cervical mucosa.**

(n=2-6

Fig. 3 - Tissue-associated MIV-150 inhibits *ex vivo* SHIV-RT infection of vaginal and cervical mucosa. MZCL (n=5) or LNG (n=4) IVRs were inserted for 24h. Vaginal and ectocervical biopsies were collected immediately after IVR removal (Post) and at the baseline. Non-stimulated vaginal (n=2-6) and cervical (n=2-3) explants were challenged with SHIV-RT (10^4 $TCID_{50}$/explant) for ~18h, washed and cultured for 14d with IL2. Infection was monitored and analyzed as in Fig.????. Shown are (**A**) SIV *gag* copies/ml of each animal (Mean of replicates ± SEM; symbols match those shown in panel B) and (**B**) SIV *gag* CUM analyses of Log_{10} transformed data (Mean ± SEM) in vaginal tissues. Each symbol represents an individual animal (MZCL group: closed symbols; LNG group: open symbols). Shown are (**C**) SIV *gag* copies/ml of each animal (Mean of replicates ± SEM; symbols match those shown in panel D) and (**D**) SIV *gag* SOFT and CUM analyses of Log_{10} transformed data (Mean ± SEM) in cervical tissues. Each symbol represents an individual animal (MZCL group). Significant *p*-values of <0.0001(***) and <0.001(**) are indicated. Input SIV *gag* copy numbers (Mean; post washout after challenge) are shown as dotted lines.

Table 1. MIV-150 and LNG concentrations in different compartments 24h post IVR insertion.

IVR	Animal ID	MIV-150			LNG
		VF (ng/ml)	Plasma (ng/ml)	Vag tissue (ng/mg)	Serum (pg/ml)
LNG IVR	FH61	nd	nd	nd	2020
	DK37	nd	nd	0.0291	3180
	GV86	nd	nd	0.0359	4637
	EM09	nd	nd	0.0301	4376
MZCL IVR	ED86	104.944	0.4862	51.2548	3480
	EJ67	87.615	0.3795	8.1234	3413
	EJ98	359.434	0.3524	31.1456	4376
	EN78	282.67	0.4801	6.9605	3761
	FC79	320.066	0.5392	6.8829	4035

nd - not detected

V. DISCUSSION AND CONCLUSIONS

DISCUSSION

PC-1005 anti SHIV-RT and HSV-2 activity in macaque explants

This study introduced *ex vivo* macaque vaginal SHIV-RT/HSV-2 co-infection and rectal SHIV-RT infection models. These models were used to test PC-1005's activity. Methodological aspects of monitoring tissue infection *ex vivo* were explored and sensitivity of SIV *gag* one-step qRT-PCR and p27 ELISA methods to monitor SHIV-RT infection in vaginal and rectal explants were compared.

A study by Rollenhagen et al. (Rollenhagen, Lathrop et al. 2014) demonstrated enhancement of *ex vivo* cervical HIV-1 infection in the HSV-2 co-infection setting. The elevated tissue HIV-1 infection coincided with increased numbers of $CD4^+CCR5^+CD38^+$ T cells and reduced anti-HIV-1 activity of low dose Tenofovir (1 µg/ml) (Rollenhagen, Lathrop et al. 2014).

In contrast to data in human cervical tissue, no enhancement of SHIV-RT infection by HSV-2 in stimulated macaque vaginal mucosa was seen in the current study. The same result was obtained when unstimulated tissues were co-challenged with the same viral doses (not shown). It is worth pointing out that that in this study and in the human ectocervical tissue co-infection model (Villegas, Calenda et al. 2016) a high HSV-2 viral challenge dose (10^6 pfu/explant) was used to assure reproducible HSV-2 infection and to test PC-1005 activity under stringent conditions. Of note, 10^6 pfu (~10^7 copies of DNA) HSV-2 per explant highly exceeds the amount of HSV-2 shed in genital fluids of HSV-2 positive patients (Mark, Wald et al. 2010; Tronstein, Johnston et al. 2011). Infection with or exposure to HSV-2 can induce apoptosis and impair DCs and T cells (Peretti, Shaw et al. 2005; Vanden Oever and Han 2010; Stefanidou, Ramos et al. 2013), potentially affecting tissue susceptibility to SHIV-RT. Although we cannot exclude these effects of HSV-2 in these tissue models, SHIV-RT infection after co-challenge of vaginal and rectal tissue was robust and reproducible. A more physiologically relevant, low-dose SHIV-RT/HSV-2 co-challenge model would be needed to explore whether and how co-infection drives mucosal SHIV-RT infection in macaques. We were unable to infect rectal mucosa with HSV-2 *ex vivo*. *In vivo* rectal HSV-2 infection was previously reported in 9 out of 11 SIV-infected macaques that were challenged rectally with $2x10^8$ pfu HSV-2 (Martinelli, Tharinger et al. 2011). However, in naïve animals, the frequency of infection after the same dose HSV-2 challenge is 55% (Guerra-Perez, Aravantinou et al. 2016) . As epithelial cells represent the primary target for HSV-2, loss of the single layer columnar epithelium during the culture period (Abner, Guenthner et al. 2005) could have contributed to the lack of *ex vivo* infection in rectal biopsies.

The side-by-side comparison indicates that one-step SIV *gag* qRT-PCR utilizing tissue culture supernatants can be used as an alternative to p27 ELISA to monitor tissue infection and product activity. The data indicate similar SHIV-RT growth kinetics and MZC activity as detected by both assays. The one-step SIV *gag* qRT-PCR has proven to be a more sensitive method than p27 ELISA. These results are in agreement with the findings of Janocko et al.(Janocko, Althouse et al. 2015), who demonstrated the feasibility of qRT-PCR as a readout of HIV infection (Janocko, Althouse et al. 2015). Also in agreement with this report (Janocko, Althouse et al. 2015), qRT-PCR did not shorten the time to detect evidence of infection. Overall, the qRT-PCR approach offers increased sensitivity and high dynamic range. The assay requires only a small volume of the supernatant (5 µl), is time and cost effective.

PC-1005 gel protected against SHIV-RT at 1:100 (~0.18 µg/ml MIV-150) and 1:300 (~0.06 µg/ml MIV-150) dilutions and against HSV-2 at 1:100 dilution in vaginal mucosa. The activity against HSV-2 was CG-mediated. Similarly, the gel at 1:100 dilution also protected against SHIV-RT in rectal mucosa. It is important to note that previous studies demonstrated that the combination of CG and zinc acetate results in antiviral synergy against HSV-2 (Fernandez-Romero, Abraham et al. 2012). The HSV-2 mouse model has shown that under stringent conditions the combination of CG and zinc protects significantly while CG alone does not protect against HSV-2 infection (Fernandez-Romero, Abraham et al. 2012; Kenney, Rodriguez et al. 2013; Kizima, Rodriguez et al. 2014). The use of undiluted formulations in a mouse model allowed to appreciate the advantage of the CG/zinc combination when compared to CG alone. In our explant system CG alone provides strong inhibition (even after diluting the gel) that masks zinc's contribution.

PC-1005 and CVLs anti HIV-1 and HSV-2 activity in human explants

Consistent with recent data demonstrating potent activity of PC-1005 against SHIV-RT infection in macaque vaginal and rectal explants (Barnable, Calenda et al. 2014; Barnable, Calenda et al. 2015; Jean-Pierre, Barnable et al. 2015; Calenda, Villegas et al. 2016), we demonstrate potent activity of PC-1005 against HIV-1_{BaL} in a single challenge and in a stringent HIV-1_{BaL}/HSV-2 co-challenge model in human ectocervical explants.

As mentioned above and similar to the previous report (Rollenhagen, Lathrop et al. 2014), we show that co-infection of ectocervical tissues with HSV-2 enhances HIV-1_{BaL} infection. However, the ~60% frequency of productive HIV-1_{BaL} infection in the presence of HSV-2 (vs. 100% following single HIV-1_{BaL} challenge) can potentially be due to apoptosis of T cells induced by exposure to high HSV-2 challenge dose in this model (Vanden Oever and Han 2010). PC-1005 at 1:100 and 1:300 dilutions (184.2 ng/ml and 61.4 ng/ml of MIV-150, respectively) strongly inhibited HIV-1 (>99%) in both single HIV-1_{BaL} and co-infection experiments, despite the significantly higher replication of HIV in the latter.

PC-1005 also provided significant CG-mediated anti-HSV-2 activity. Although anti-HSV-2 activity was lost at the high gel dilution (1:300), it may still be evident *in vivo* against more physiologically relevant HSV-2 inoculum (Mark, Wald et al. 2010; Tronstein, Johnston et al. 2011). Of note, tenofovir at low concentrations (1 µg/ml) was shown to inhibit single infections with HIV-1_{BaL} and HSV-2, but a higher tenofovir dose (100 µg/ml) was required to inhibit infections with both viruses in co-challenge experiments (Rollenhagen, Lathrop et al. 2014).

In support of earlier data demonstrating that anti-HIV-1 (cell-based assays), anti-SHIV-RT (macaque vaginal explants), anti-HSV-2 (murine model) activity of PC-1005 and anti-HPV activity (murine model) of CG is maintained in the presence of biological fluids (SP) (Barnable, Calenda et al. 2014; Kizima, Rodriguez et al. 2014; Rodriguez, Kleinbeck et al. 2014; Fernandez-Romero, Deal et al. 2015) (Kenney et al, in preparation), PC-1005 was active against HIV-1_{BaL} and HSV-2 in human ectocervical explants in the presence of vaginal fluids. Infections levels inversely correlated with MIV-150 levels (HIV-1) and CG (HSV-2) levels in the CVLs. The design of these experiments (tissues pre-incubation with CVLs followed by washout and then challenge with HIV-1 or co-challenge with HIV-1/HSV-2) offered rigorous testing conditions against both viruses. Therefore, here we introduced new models for testing anti-HIV and anti-HSV activity of CVLs in relevant mucosal targets.

This study has several caveats, including caveats associated with the use of cervical explant models (Anderson, Pudney et al. 2010). Also, the study does not address activity of PC-1005 in human vaginal mucosal targets due to difficulties obtaining vaginal surgical tissues. As expected based on the dose-dependent effect of CG against SHIV-RT *ex vivo* (Barnable, Calenda et al. 2014) in macaque explants and dose-dependent and time-dependent effects *in vivo* (Turville, Aravantinou et al. 2008; Kenney, Aravantinou et al. 2011; Kenney, Singer et al. 2012) in macaques, CG at high concentrations (300 µg/ml, 1:100 dilution of PC-525) inhibited HIV-1_{BaL} infection in the single challenge model. This was not the case in the co-infection model (HIV-1/HSV-2), where HIV replication was significantly more robust. Moreover, spiking CVLs with CG provided anti-HIV-1_{BaL} activity; however, the inhibitory activity of CVLs containing PC-1005 components was more potent, pointing to MIV-150-mediated protection. We were not able to test activity of tissue-associated MIV-150 and CG post gel application *in vivo* as vaginal and cervical biopsies in the Phase 1 PC-1005 study were prioritized for histological evaluation. Lastly, the small number of available CVLs provided little statistical power. In spite of this hindrance, the fact that we observed significant anti-HIV and anti-HSV-2 activities of CVLs is striking; and, as noted earlier, with the decision to minimize the type 1 error rate, hints at the likelihood that this is not a spurious finding.

IVRs

This study demonstrates that MIV-150 released from the MZC/MZCL IVRs *in vivo* (VF and/or tissue-associated) is highly effective against *ex vivo* SHIV-RT infection in vaginal and cervical mucosa. LNG did not compromise MIV-150 activity. In fact, SOFT (but not CUM) analysis revealed that VF from MZCL IVR animals more strongly inhibited SHIV-RT relative to VF from MZC IVR animals. MIV-150 in VF from the prototype MZC and MZCL IVRs provided dose-dependent inhibition of *ex vivo* SHIV-RT infection in macaque vaginal explants. MIV-150 potently inhibits SHIV-RT in PBMCs. >20 nM MIV-150 in the VF from MZCL IVRs (>10 fold EC_{90} in cell-based assays) strongly reduced infection, pointing to higher concentration of MIV-150 needed for SHIV-RT inhibition in explants vs. cell-based assays. This data are consistent with our previous report (Ouattara, Barnable et al. 2014). The results agree with our earlier reports documenting the activity of MIV-150 in cells and explants in the presence of VF (Singer, Mawson et al. 2012; Ouattara, Barnable et al. 2014).

Previous PK and efficacy studies of MZC gel demonstrated that tissue MIV-150 concentrations following gel application better predict efficacy against vaginal SHIV challenge than plasma or VF MIV-150 concentrations (Kenney, Aravantinou et al. 2011). Similarly, the NNRTI dapirivine showed concentration-dependent inhibition of HIV in cervical tissue post IVR exposure (Chen, Panther et al. 2015). Tenofovir (TFV) concentrations in vaginal lymphocytes post gel application in macaques predicted efficacy against vaginal SHIV challenge better than plasma TFV levels (VF levels of TFV were not measured) (Parikh, Dobard et al. 2009; Dobard, Sharma et al. 2012; Romano, Kashuba et al. 2013). PK/PD assessments in humans identified that TFV concentrations in VF and detection of TFV in plasma (CAPRISA 004) were associated with reduced HIV infection (Karim, Kashuba et al. 2011; Romano, Kashuba et al. 2013). These data highlight the need to have detailed assessment of compartmental PK/PD in animal models (measuring PK and PD at the same time) to inform efficacy studies and clinical trial design.

This study provides important insights into plasma, tissue and VF MIV-150 concentrations and tissue PD relationships. Tissue-associated MIV-150 (≥6.8 ng/mg in vaginal mucosa; not measured in cervix) afforded 96% protection against *ex vivo* SHIV-RT challenge 24h post IVR exposure vs. baseline. We did not observe MIV-150 dose-dependent protection, likely because of high MIV-150 concentrations in all vaginal tissue samples. High tissue MIV-150 concentrations detected in our study are likely the result of greater release of MIV-150 from MZCL IVRs (Ugaonkar, Wesenberg et al. 2015) compared to MIV-150 IVRs (Singer, Mawson et al. 2012).

As we discussed in (Ouattara, Barnable et al. 2014), previous studies reporting tissue concentrations of MIV-150 (Singer, Mawson et al. 2012) and tissue PD (Ouattara, Barnable et al. 2014) suggest that ~0.6 ng/mg of MIV-150 in vaginal tissue protects against *ex vivo* SHIV-RT infection. Also, PK/efficacy studies suggested that even lower concentrations of MIV-150 (~0.1 ng/mg) could protect against *in vivo* vaginal SHIV-RT challenge when IVRs remain in place post

challenge (Singer, Mawson et al. 2012). However, the *in vivo* protection was lost when IVRs were removed right before the challenge (Singer, Mawson et al. 2012). MIV-150 concentrations in VF did not predict anti-SHIV-RT activity in tissues from corresponding animals. The results were somewhat expected due to the variable VF volumes in animals.

It is unlikely that CG and ZA contributed to the observed anti-SHIV-RT activity in the tissue PD studies. Similar to the recent report (Ugaonkar, Wesenberg et al. 2015), no CG was detected in macaque VF 24h post IVR insertion. VF ZA was not measured in our study as VF ZA levels were reported below the LLOQ of the assay (15 µg/ml) in a previous study (Ugaonkar, Wesenberg et al. 2015). We previously demonstrated no effect of single exposure to ZA at 466 µM (102 mg/ml) on SHIV-RT infection in vaginal explants (Barnable, Calenda et al. 2014). The small number of animals (n=4-5 per group) in API concentrations and tissue PD studies represents a limitation. These studies are exploratory in nature and larger powered studies are needed to confirm these results.

It has been previously discussed that increased ectocervical tissue infection post-Depo Provera (long-acting reversible hormonal contraceptive birth control progestin) treatment could have resulted in a lack of infection inhibition by MIV-150 in the ectocervix (Ouattara, Barnable et al. 2014). It is unknown if LNG changes mucosal immune milieu and susceptibility to HIV after short-term exposure. As signaling through steroid receptors is rapid (Levin 2008) and can potentially lead to changes in mucosal susceptibility to HIV, we chose to include LNG IVR control group for MZCL IVR *in vivo* studies. Similar to short-term *in vitro* LNG exposure experiments, *in vivo* exposure to LNG in non-Depo treated animals did not change vaginal tissue infection levels. As the number of ectocervical samples in LNG group was limited, we cannot conclude whether infection in cervical mucosa was affected by LNG. Long-term exposure to LNG in LNG IUD users was reported to lead to contrasting changes in the expression of CCR5 on peripheral blood CD4 and CD8 T cells and on endometrial and cervical T cells; changes in tissue (endometrium and cervix) gene expression mediating cell homing, cell-cell signaling and immune activation (Achilles, Creinin et al. 2014; Goldfien, Barragan et al. 2015; Sciaranghella, Wang et al. 2015). Use of combined oral contraceptives containing LNG or insertion of Norplant leads to decrease in epithelial thickness in macaques (Hild-Petito, Veazey et al. 1998; Dietz Ostergaard, Butler et al. 2015) and may enhance susceptibility to HIV. Furthermore, LNG was recently reported to decrease genital epithelial barrier function, induce influx of inflammatory cells to the mucosa and increase susceptibility to HSV-2, which is known to increase HIV transmission (Glynn, Carael et al. 2001; Freeman, Weiss et al. 2006; Sobngwi-Tambekou, Taljaard et al. 2009; Barnabas and Celum 2012). New models involving low viral challenge dose and shorter vs. longer-term exposure to LNG would be useful to address effects of LNG on mucosal tissue susceptibility to HIV/SHIV-RT.

CONCLUSIONS

The results demonstrating potent activity of PC-1005 against co-infection of macaque vaginal and human ectocervical mucosa with HIV or SHIV-RT and HSV-2, and the anti SHIV-RT in macaque rectal mucosa add to the previously published data showing potent PC-1005 activity against single cell-free or cell-associated SHIV-RT challenge of macaque vaginal mucosa (Barnable, Calenda et al. 2014; Barnable, Calenda et al. 2015). Furthermore these findings are consistent with the extensive *in vitro*, *ex vivo* (explants) and *in vivo* (mice, macaques) data, demonstrating efficacy of PC-1005 gel against HIV, SHIV-RT and HSV-2 (Kenney, Aravantinou et al. 2011; Kenney, Singer et al. 2012; Hsu, Aravantinou et al. 2013; Barnable, Calenda et al. 2014; Kenney, Derby et al. 2014; Kizima, Rodriguez et al. 2014; Barnable, Calenda et al. 2015; Jean-Pierre, Barnable et al. 2015). PC-1005 is the only microbicide gel currently being tested in clinical trials that targets HIV and two other main STIs (HSV-2 and HPV) (Fernandez-Romero, Deal et al. 2015). Overall, the data presented in this study point to the potent anti-HIV and anti-HSV-2 activity of PC-1005 in mucosal targets and endorse the further development of PC-1005 as a broad-spectrum on-demand microbicide.

Moreover, this study shows that prototype MZCL IVRs release active MIV-150 *in vivo* within 24h hour of insertion and that MIV-150 protects vaginal and cervical mucosa against *ex vivo* challenge with SHIV-RT, and LNG does not interfere with this activity. These findings indicate that the MZCL IVR is a viable MPT IVR candidate. An MZCL IVR that simultaneously prevents the sexual transmission of HIV, HSV-2, HPV, and unintended pregnancy will have the potential to significantly reduce the worldwide incidence of HIV and improve the sexual and reproductive health of millions of women.

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