Differential effects of combinations of anti-retrovirals and progestins on gene expression and HIV-1 replication in cells of the Female Genital Tract

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Supervisor(s): Prof. Janet Patricia Hapgood and Dr Michelle Maritz
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Signed by candidate

Signature:.......................................................... Date:......03/August/2017..........
Abstract

Young women in areas of high HIV risk are more vulnerable to new HIV infections compared to men. With the development of programmes using anti-retrovirals (ARVs) to prevent HIV infection, the concurrent use of hormonal contraception and ARVs is likely to increase in young women. Products delivering ARVs and hormonal contraceptives intravaginally, are in development, and aim to simultaneously prevent unintended pregnancies and prevent HIV infection. However, little is known about the combinatorial effects of ARVs and hormonal contraceptives in the female genital tract. The mucosal surface in the female genital tract is the initial site of HIV entry and viral replication. Understanding the effect of hormonal contraceptives and ARVs is relevant to the potential safety of these for long term use.

The present study aims to investigate the effects of the ARVs dapivirine (DPV), tenofovir disoproxil fumarate (TDF) and a panel of progestins, alone and in combination with each other, on the expression of immune function genes relevant to HIV acquisition, on steroid receptor activity, and on HIV replication. Analysis of expression of select pro- and anti-inflammatory genes revealed that, DPV exerts gene-specific pro-inflammatory and cytotoxic effects on cervical cells and tissue. Co-stimulation with the progestin medroxyprogesterone acetate (MPA) revealed that MPA can potentiate and inhibit the DPV-induced pro-inflammatory effects, in a gene-specific manner in cervical cells. DPV was shown to alter the efficacy and potency of an androgen receptor (AR) ligand, demonstrating its ability to influence AR activity. Furthermore, DPV inhibited viral replication but also increased HIV co-receptor expression in the absence and presence of MPA. In contrast to DPV, TDF had no effect on the expression of pro-inflammatory immune function genes or HIV co-receptors in the absence and presence of MPA. TDF had no effect on endogenous steroid receptor levels in cervical cells and showed no significant effects on steroid receptor transactivation.

In summary, data from the present study show that TDF may be the better choice of ARV for combinatory usage with hormonal contraceptives. An association between genital inflammation and HIV acquisition has previously been established and thus the potentiated pro-inflammatory effects of DPV may potentially change the microenvironment of the female genital tract to favour increased risk of HIV or other genital tract infections, when used alone and in combination with hormonal contraceptives such as MPA. Furthermore, DPV’s influence on steroid receptor activity points towards potential for increased side effects due to off-target AR effects. These results have serious implications for women using hormonal contraception who may consider the use of DPV as a microbicide. However, whether in vitro results will occur in vivo, remains to be established.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AP-1</td>
<td>activator protein 1</td>
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<td>AR</td>
<td>androgen receptor</td>
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<td>ARV</td>
<td>anti-retroviral</td>
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<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CAPRISA</td>
<td>Centre for the AIDS Programme of Research in South Africa</td>
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<tr>
<td>CCL</td>
<td>chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR5</td>
<td>chemokine receptor type 5</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
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<td>CRI</td>
<td>co-receptor inhibitor</td>
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<tr>
<td>CS-FCS</td>
<td>charcoal stripped fetal calf serum</td>
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<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<tr>
<td>DEX</td>
<td>dexamethasone</td>
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<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
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<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagles medium</td>
</tr>
<tr>
<td>DMPA</td>
<td>depo-medroxyprogesterone acetate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DPV</td>
<td>dapivirine</td>
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<td>E2</td>
<td>estradiol/estrogen</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EC50</td>
<td>effective concentration required for 50% of maximal response</td>
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<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
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<td>EE</td>
<td>ethyl estradiol</td>
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<td>EFV</td>
<td>efavirenz</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Env</td>
<td>HIV envelope protein</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<td>ETG</td>
<td>etonogestrel</td>
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<td>EtOH</td>
<td>ethanol</td>
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<td>FDA</td>
<td>U.S Food and Drug Administration</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>FGT</td>
<td>female genital tract</td>
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<td>FI</td>
<td>fusion inhibitor</td>
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<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>FTC</td>
<td>emtricitabine</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>GILZ</td>
<td>glucocorticoid induced leucine zipper</td>
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<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
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<tr>
<td>HAART</td>
<td>highly active anti-retroviral therapy</td>
</tr>
<tr>
<td>HC</td>
<td>hormonal contraception</td>
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<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus subtype 1</td>
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<tr>
<td>HIVR4P</td>
<td>HIV research for prevention conference</td>
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<tr>
<td>HPV</td>
<td>human papilloma virus</td>
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<tr>
<td>hrs</td>
<td>hours</td>
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<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
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<tr>
<td>IκBα</td>
<td>Inhibitor kappa B alpha</td>
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<tr>
<td>IC₅₀</td>
<td>inhibitory concentration that reduces the maximal response by half</td>
</tr>
<tr>
<td>IHC</td>
<td>injectable hormonal contraceptives</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>INI</td>
<td>integrase inhibitor</td>
</tr>
<tr>
<td>IPM</td>
<td>International Partnership for Microbicides</td>
</tr>
<tr>
<td>IUD</td>
<td>intrauterine device</td>
</tr>
<tr>
<td>IVR</td>
<td>intravaginal ring</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<tr>
<td>LH</td>
<td>luteinising hormone</td>
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<tr>
<td>LNG</td>
<td>levonorgestrel</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MOPS</td>
<td>4-morpholine-propanesulfonic acid</td>
</tr>
<tr>
<td>MPA</td>
<td>medroxyprogesterone acetate</td>
</tr>
<tr>
<td>MPT</td>
<td>multipurpose prevention technology</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>MTN</td>
<td>Microbicide Trials Network</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>NES</td>
<td>nesteronone</td>
</tr>
<tr>
<td>NET</td>
<td>norethisterone</td>
</tr>
<tr>
<td>NET-A</td>
<td>norethisterone acetate</td>
</tr>
<tr>
<td>NET-EN</td>
<td>norethisterone enanthate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NNRTI</td>
<td>non-nucleotide reverse transcriptase</td>
</tr>
<tr>
<td>NRTI</td>
<td>nucleotide reverse transcriptase</td>
</tr>
<tr>
<td>P₄</td>
<td>progesterone</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>protease inhibitor</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PrEP</td>
<td>Pre-exposure prophylaxis</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse transcription quantitative PCR</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated upon activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RBA</td>
<td>relative binding affinity</td>
</tr>
<tr>
<td>RLU</td>
<td>relative light units</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
</tr>
<tr>
<td>SR</td>
<td>steroid receptor</td>
</tr>
<tr>
<td>SRE</td>
<td>steroid response element</td>
</tr>
<tr>
<td>STI</td>
<td>sexually transmitted infection</td>
</tr>
<tr>
<td>TBS</td>
<td>TRIS-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TRIS-buffered saline-tween</td>
</tr>
<tr>
<td>TDF</td>
<td>tenofovir disoproxil fumarate</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>UN</td>
<td>United Nations</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>Joint United Nations Programme on HIV/AIDS</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per unit volume</td>
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<tr>
<td>v/v</td>
<td>volume per unit volume</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>VOICE</td>
<td>Vaginal and Oral Interventions to Control the Epidemic</td>
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4.5 References

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Chapter 1: Literature review

1.1 Impetus for multipurpose prevention strategies against HIV and unintended pregnancies

HIV/AIDS is still one of the deadliest diseases in the world, alongside malaria and tuberculosis, with 2.1 million new infections occurring worldwide in 2015 (WHO, 2016). As most infections occur through heterosexual intercourse, young women aged between 15-24 are at a very high risk of HIV infection (UNAIDS, 2016). Sub-Saharan Africa is the region most impacted with the disease and here women account for 56% of new infections among adults (UNAIDS, 2016). The disproportional effect seen between men and women is influenced by a combination of factors including greater physiological risks in women, and imbalanced gender norms in the area (Mabala, 2006, Ramjee and Daniels, 2013). Most women are unable to negotiate safer sex or ensure their partners’ faithfulness, so they largely depend on partner cooperation to protect themselves from HIV and other sexually transmitted infections (STIs) (Higgins et al., 2010, Jewkes et al., 2010). Other factors such as poverty, malnutrition and a lack of education, also impact the risk of HIV in women.

Young women in Sub-Saharan Africa are also impacted by high rates of unintended pregnancies, and as a result, the use of hormonal contraceptives has increased in the area. This has led to reduced rates of infant mortality and maternal mortality that are prevalent in cases of unintended or closely-spaced pregnancies. Long-acting injectable hormonal contraceptives (IHC) are the most widely used method of contraception and account for 46% of hormonal contraceptive usage in Southern Africa (Pacqué-Margolis et al., 2013). Approximately 11 million women use IHCs in Sub-Saharan Africa (UN, 2013).

The increased prevalence of unintended pregnancies, infant and maternal mortality combined with the high prevalence of HIV in Sub-Saharan Africa, increases the need for young women to avoid getting pregnant without increasing their risk of HIV infection (Ralph et al., 2015b). However, a correlation between IHC use and HIV prevalence has previously been established, although this does not prove a causal relationship between the two (Butler et al., 2013). In recent years, a growing number of clinical and molecular studies have shown strong evidence for the association of the main IHC used in Sub-Saharan Africa known as Depo-Provera or medroxyprogesterone acetate (MPA), with increased risk of HIV acquisition (Polis et al. 2016). This has encouraged researchers to look at alternative long-term contraceptive methods, which are effective in preventing unplanned pregnancies, and are not associated with an increased risk of HIV acquisition.
In an effort to provide women with more control over their health, the development of multipurpose prevention technologies (MPTs) that protect women from HIV infection and unplanned pregnancies, and require no partner cooperation, is a priority. Currently the only MPT available to women is the condom, and this method still has the disadvantage of requiring partner cooperation. New MPTs, which are women-controlled and discreet, can empower women with a wider choice in prevention (Thurman et al., 2013). The MPTs that are currently being developed deliver a hormonal contraceptive and an anti-retroviral (ARV) drug simultaneously, and target the female genital tract mucosa directly through methods such as intravaginal rings and topical gels.

1.2 Structure and function of progestins in hormonal contraceptives

The two main methods for hormonal contraception are combined hormonal contraception and progestin-only hormonal contraception. These contraceptives are delivered by a wide variety of methods ranging from oral, intramuscular, trans-epidermal and intravaginal. Progestogens are collectively defined as any natural or synthetic steroid hormone, which prevents ovulation in preparation for pregnancy by changing the estrogen-primed endometrium of the uterus from a proliferative state to a secretory state (Stanczyk et al., 2012). The naturally occurring progestogen in women is progesterone (P₄). The concentration of P₄ determines whether ovulation will occur. When P₄ is high, ovulation is prevented, and when it is low, ovulation is induced. The term progestin is used to describe synthetic progestogens that are designed to mimic the action of P₄, and as for P₄, their progestogenic effects are mediated by the progesterone receptor (PR) (Hapgood et al., 2014a, Stanczyk et al., 2012).

The panel of progestins used in this study include the first generation progestins MPA and norethisterone (NET), as well as levonorgestrel (LNG), nestorone (NES) and etonogestrel (ETG). These progestins vary widely in their chemical structures and are commonly grouped into two categories: those that are structurally related to P₄, known as pregnanes and norpregnanes, and those that are structurally related to testosterone, known as estranes and ethyl-gonanes. This grouping does not imply that the progestins are derived from these molecules. MPA and NES are structurally similar to P₄, while NET, LNG and ETG are more structurally similar to testosterone (Stanczyk et al., 2012). Structures and biological properties of progestins, including their method of administration, serum concentrations and their binding affinities for steroid receptors are depicted in Table 1.

MPA is a first generation pregnane progestin, that is administered as a three-monthly intramuscular (Depo-Provera®, DMPA-IM) or subcutaneous injection (Sayana® Press, DMPA-SQ), and has been widely used as a contraceptive for many decades. DMPA-IM is given at a
Dose of 150 mg and reaches peak serum concentrations at an average of 21 nM (Bonny et al., 2014, Fotherby et al., 1980, Nanda et al., 2008, Ortiz et al., 1977). DMPA-SQ is given at a lower dose of 104 mg and studies that have investigated its pharmacokinetic parameters in women, have found that it has lower peak serum concentrations, ranging from 2.5 - 4 nM (Halpern et al., 2014, Jain et al., 2004, Toh et al., 2004). Despite its widespread usage, DMPA has been associated with many adverse effects such as bone mineral density loss, weight gain and increased breast cancer risk (Beksinska et al., 2010, Li et al., 2012, Lopez et al., 2015). Over the past few years, several clinical observational studies have investigated the associated risk of HIV acquisition with DMPA use. The most recent meta-analysis data revealed that there is a hazard ratio of 1.5 with DMPA use, compared to women not using hormonal contraception (Polis et al., 2016a); however, this observed increase is still controversial due to possible confounding factors such as, sexual exposure and condom usage, that are difficult to adjust for in observational studies (Murphy et al., 2014). The possible associated risk of infection has led to much concern about the safety of use of DMPA in areas of HIV-1 risk, and alternative progestin contraceptive options are being considered. In light of this, important questions have arisen including whether any of the other progestin containing hormonal contraceptives pose a risk for HIV acquisition, and whether there are any safer options, which can be used as an alternative to MPA in areas of high HIV prevalence, like Sub-Saharan Africa.

Another first generation estrane progestin is NET. The administered compound is norethisterone enanthate (NET-EN), which has a longer side chain group (R=OCO(CH₂)₅CH₃), compared to the hydroxyl group side chain of NET. It is administered as a two-monthly injectable (Hapgood et al., 2004, Stanczyk and Roy, 1990), marketed as Noristerat and given at a 200 mg dose. The biologically active compound, NET, reaches peak serum concentrations between 10 - 50 nM (Goebelsmann et al., 1979, Sang et al., 1981). There are limited studies available that have investigated the risk of HIV acquisition with NET-EN usage. Studies available have compared NET-EN, DMPA and oral contraceptive use versus non-hormonal contraceptive use and have found that NET-EN shows no significant increased risk of HIV acquisition compared to DMPA and non-hormonal contraceptive use (Brind et al., 2015, Morrison et al., 2015, Polis et al., 2016a, Ralph et al., 2015a). Since NET-EN is used mostly in Africa, administered similarly to MPA, and has not been found to be associated with an increased risk of HIV acquisition, it is a good candidate to be an alternative for DMPA in Sub-Saharan Africa.

NES is a 19-norpregnane progestin and is currently being developed as a one-year vaginal ring in combination with ethyl estradiol (EE) (Huang et al., 2015). The ring is designed to
release 150 μg NES and 15 μg EE daily, and prevent pregnancy when used continuously on a 21-day-in/7-day-out cyclic regimen. Peak serum concentrations of NES are approximately 0.3 nM (Sivin et al., 2005). Currently there are no data available showing the association of NES with increased HIV risk, and it has been shown to have no effect on normal vaginal flora and vaginal bacterial infections (Huang et al., 2015).

LNG is an ethyl-gonane progestin that can be administered as an intrauterine device (IUD), an oral pill in combination with EE, or an implant that can last up to 5 years (e.g Jadelle®) (Meirik et al., 2001, Stanczyk et al., 2012). Depending on the method of administration used, LNG can reach peak serum concentrations ranging from 0.3 - 28 nM (Hidalgo et al., 2009, Licea-Perez et al., 2007, Sivin et al., 1997). There currently are limited reports on the risk of HIV acquisition with LNG use; however, findings to date have shown no significant association between LNG and HIV acquisition (Baeten et al., 2007, Lavreys et al., 2004, Wall et al., 2015).

ETG is the active metabolite of desogestrel which, like LNG, is an ethyl-gonane (Stanczyk et al., 2012). ETG is administered as a combined oral contraceptive, or an implant, or as an intrauterine device in combination with EE. The intrauterine device and the implant can last for periods of one to three years (e.g Nexplanon®). Peak serum concentrations range from 0.8 - 7.5 nM (Croxatto et al., 1999, Kuhnz et al., 1992, Thomas et al., 2013, Wenzl et al., 1998). There are currently no reports available associating ETG use as an implant or an IUD, with HIV acquisition.

Although the progestins described are designed to mimic the action of P₄ and produce their progestogenic effects via the PR, they do, however, exhibit other biological effects that differ from that of progesterone. These non-progestogenic effects are proposed to occur through off-target effects of the progestins binding to other receptors besides the PR, namely the glucocorticoid receptor (GR), the androgen receptor (AR), or the mineralocorticoid receptor (MR) (Africander et al., 2011, Stanczyk et al., 2012).
### Table 1: Structure and biological properties of progestins

<table>
<thead>
<tr>
<th>Progestin</th>
<th>Method of administration</th>
<th>Peak Serum concentration (nM)</th>
<th>Reference</th>
<th>Binding Affinities (% RBA)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NET</td>
<td>2 monthly intramuscular injection</td>
<td>10 - 50</td>
<td>Goeboelsmann et al. 1979; Sang et al. 1981</td>
<td>PR: 27-34 GR: 0.8 AR: 134 MR: 0.15 ER: -</td>
</tr>
<tr>
<td>LNG</td>
<td>1 year intra-uterine device (IUD)</td>
<td>0.3 - 28 ( Depending on type used)</td>
<td>Hidalgo et al. 2009; Licea-Perez et al. 2007; Sivin et al. 1997</td>
<td>PR: 23-96 GR: 1-7.5 AR: 58 MR: 17-75 ER: -</td>
</tr>
<tr>
<td>NES</td>
<td>1 year vaginal ring used in combination with estradiol</td>
<td>0.3</td>
<td>Sivin et al. 2005</td>
<td>PR: 110 GR: 38 AR: 0.2 MR: - ER: -</td>
</tr>
<tr>
<td>ETG</td>
<td>1 year intra-uterine device (IUD)</td>
<td>0.8 - 7.5 ( Depending on type used)</td>
<td>Croxatto et al. 1999; Kuhnz et al. 1992; Thomas et al. 2013; Wenzl et al. 1998</td>
<td>PR: 150 GR: 14 AR: 20 MR: &lt;0.1 ER: -</td>
</tr>
</tbody>
</table>

Notes: * Percentage relative binding affinities (%RBA) were obtained from Hapgood, Africander et al. 2014 and are given as relative to 100% for reference steroids which were as follows: PR, progesterone; AR, dihydrotestosterone; GR, dexamethasone; MR, aldosterone (Hapgood et al., 2014a). (-) no interaction

### 1.3 Molecular mechanisms of action of hormonal contraceptives via steroid receptors

#### 1.3.1 Ligand binding affinities

Progestins have high affinity for the PR; however, they also bind different receptors with varying affinities, compared to their affinity for the PR. These are summarised in Table 1 (Africander et al., 2013, Africander et al., 2011, Africander et al., 2014, Kuhl, 2011, Ronacher et al., 2009, Stanczyk, 2003, Stanczyk et al., 2012).
MPA has a relatively high affinity for the GR, compared to NES and ETG, which have a lower affinity for the GR. MPA is also shown to have higher binding affinity for the GR than the endogenous GR agonist, cortisol (Kontula et al., 1983). LNG and NET have very low affinities for the GR, but do exhibit relatively high affinity for the AR, as does MPA. MPA and NET were also shown to have similar binding affinities for the AR as DHT, the natural agonist of the AR (Africander et al., 2014). LNG has a higher binding affinity for the MR compared to the other progestins while all have been shown not to bind to the estrogen receptor (ER) (Stanczyk et al., 2012).

### 1.3.2 Transactivation and transrepression

Although progestins can bind to receptors other than the PR, this may not always reflect their biological activity via different receptors. At the cellular level, steroid receptors function as ligand activated transcription factors, which can alter the expression of specific genes in target cells (Huang et al., 2010). They can either activate or repress the transcription of genes, known as transactivation or transrepression, respectively. Transactivation occurs when a steroid hormone enters the cell, binds to the steroid receptor in the cytoplasm, causing it to dimerise and translocate to the nucleus. Once there, it interacts directly with specific cis elements on the promoter region of the target gene called steroid response elements (SRE) and this interaction causes increased transcription of the gene (Africander et al., 2011). In contrast, steroid receptors negatively regulate the transcription of genes through different mechanisms of transrepression. Transrepression mechanisms include direct binding of the receptor to negative SREs, and tethering of the receptor with other transcription factors that are bound to the DNA of the target gene, such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) (Newton and Holden, 2007, Petta et al., 2016, Surjit et al., 2011). Transactivation and transrepression mechanisms of the GR are depicted in Figure 1.1.

### 1.3.3 Crosstalk of hormonal contraceptives with different steroid receptors

MPA has been shown to have glucocorticoid activity in animal models and studies have shown that it can alter gene expression via the GR in vitro (Ronacher et al., 2009). A study by Govender et al. showed that MPA, but not NET-A and P₄ increases mRNA expression of the anti-inflammatory genes GILZ and IκBα, and decreases mRNA expression of the pro-inflammatory IL-6, IL-8 and RANTES genes in endocervical cell lines via the GR (Govender et al., 2014). MPA and NET were also shown to be efficacious AR agonists, with activities comparable to that of the AR agonist DHT in in vitro transrepression and transactivation systems (Africander et al., 2014). LNG has also shown some AR activity in animal models (Phillips et al., 1990). ETG showed weak glucocorticoid activity, while NES showed no
glucocorticoid activity, despite having some binding affinity for the GR (Kuhl, 2011, Kumar et al., 2000).

Figure 1.1 Schematic of transactivation and transrepression mechanism of the GR. Upon ligand (glucocorticoid) binding to the GR, the GR undergoes a conformational change and translocates to the nucleus. Once in the nucleus liganded GR can exert positive and negative transcriptional effects via different mechanisms. In the transactivation mechanism GR occupies GRE sites of a gene promoter to activate transcription. In the transrepression mechanism, GR can bind directly to negative GRE sites (nGRE) or interact with transcription factor (TF) bound sites such as AP-1 or NF-κB sites to suppress gene transcription. Figure adapted from Beck et al. (Beck et al., 2011)

Crosstalk of progestins with other steroid receptors is largely dependent on the potency and efficacy of the progestin for the steroid receptor. Potency is defined as the concentration of ligand required for half of the maximal biological response and is referred to as the EC$_{50}$, whereas efficacy is the maximal-induced response for that particular ligand (Africander et al., 2011). Off-target effects of progestins likely occur when serum levels are in the EC$_{50}$ range of other steroid receptors. Potency and efficacies are determined using dose response curves. Due to the concerns linking MPA with increased HIV risk, studies have investigated the potency of MPA in altering gene expression via the GR. MPA was shown to have potencies in the range of 3 - 20 nM in regulating expression of GR regulated genes in cervical cell lines (Govender et al., 2014, Louw-du Toit et al., 2014). Potencies of 10 - 100 nM have also been reported for MPA in peripheral blood mononuclear cells (PBMCs), when regulating immune function genes (Hapgood et al., 2014b, Huijbregts et al., 2013).
1.4 Hormonal contraceptives and the risk of HIV

There is evidence from clinical data for several plausible biological mechanisms whereby MPA may increase HIV-1 acquisition in the female genital tract, including changing the expression of several soluble immune mediators, increasing the permeability of the genital tract to pathogens, altering the microbiome, as well as increasing the recruitment of HIV-1 target cells or expression of HIV-1 co-receptors (Murphy et al., 2014). However, further mechanistic studies are needed to determine the underlying mechanisms that link MPA to increased risk of HIV infection.

1.4.1 Effect of MPA on soluble immune mediators

Several studies have shown that MPA can directly regulate immune function mediators at concentrations around the peak levels of DMPA users. MPA was shown to inhibit cytokine production (IL-2, IL-3, IL-4, IL-5, IL-9, IL-12) by activated T cells and CD14+ cells and this effect was reversed by the GR antagonist RU486, demonstrating GR involvement (Huijbregts et al., 2013). MPA showed similar immunosuppressive effects in endocervical cells by inhibiting IL-8, IL-6, and RANTES via the GR (Govender et al., 2014, Louw-du Toit et al., 2014). In another study, MPA significantly increased the pro-inflammatory IL-12 and suppressed anti-inflammatory IL-10 expression in a dose-dependent manner in ectocervical cells (Louw-du Toit et al., 2014). MPA was also shown to increase IL-6 levels in human vaginal epithelial cells at concentrations of approximately 37 nM, while higher concentrations increased several other cytokines including TNF, IL-8, MIP-1α, and RANTES (Irvin and Herold, 2015). These studies have demonstrated the ability of MPA to exert pro- and anti-inflammatory effects on select genes via the GR, in a cell-specific manner.

1.4.2 Effect of MPA on increasing HIV target cells and HIV co-receptors

The main target cells for HIV are CD4+ T cells, macrophages, dendritic cells, B cells, and CD8+ T cells. Whether HIV replication occurs in epithelial cells is controversial (Shen et al., 2014). Previous studies have shown that MPA reduces dendritic cell expression of CD40 and CD80 molecules in PBMCs, which are essential for CD4+ and CD8+ T cell proliferation and dendritic cell activation (Quispe Calla et al., 2015). A study that investigated the effect of MPA on increasing viral replication in unstimulated PBMCs, found that stimulation of the CD3+ CD8-T cell population of PBMCs with MPA, increased HIV infection (Sampah et al., 2015). In another study investigating the association of injectable progestins with HIV risk in women in South Africa, injectable progestin users showed a higher frequency of HIV target CCR5+ CD4+ T cells, compared to women not on hormonal contraceptives, although no differences
between DMPA and NET-A users were observed, possibly due to insufficient statistical power in this comparison (Byrne et al., 2016). DMPA along with LNG use has also been associated with increased T cell CCR5 HIV co-receptor expression in women (Chandra et al., 2013, Sciaranghella et al., 2015). Smith-McCune et al. recently reported no increases of CCR5 expression in CD4+ T cells of DMPA users in the endometrium or endocervix; however, the study found that women using DMPA had higher proportions of endometrial CD4+ CD8+ T cells expressing markers of activation, compared to women not on hormonal contraceptives. DMPA use also resulted in an increase in density of macrophages in the endometrium, which are also known to be targets of HIV-1 (Smith-McCune et al., 2017). These studies highlight the ability of MPA to alter the activation, frequency and co-receptor expression of key HIV target cells, and this may provide evidence for a biological association between MPA use and increased HIV risk.

1.4.3 Effect of MPA on increasing permeability of the genital tract and altering the vaginal microbiome

The female genital tract is structurally designed to protect itself from foreign pathogens. Epithelial mucosal cells lining the upper and lower genital tract, play an important role in this defence. The upper genital lining is made up of a single layer of columnar epithelial cells that line the endometrium and ectocervix, while the lower genital lining is made up of multi-layered, squamous epithelial cells that line the vagina and ectocervix (Wira et al., 2005). These epithelial cells are held together by tight junctions and adherens proteins that regulate the movement of molecules across the epithelium, and a weakened epithelial barrier increases permeability of the genital tract to foreign pathogens (Wira et al., 2015). In animal models, studies have shown that MPA is associated with thinning of the epithelial lining, and in some studies, MPA is given to animals to make them more susceptible to HIV or simian immunodeficiency virus (SIV) infection (McNicholl et al., 2014, Vishwanathan et al., 2011). This thinning of the epithelial lining has not been observed in women. MPA and LNG were shown to increase genital permeability in a mouse model by decreasing the expression of the adhesion molecules, desmoglein 1α (DSG1α) and desmocollin (DSC1) (Calla et al., 2016b). In the same study, ectocervical biopsy tissue taken from women before and one month after initiating DMPA, revealed that DSG1α expression was also reduced, similar to the downregulation observed in mice. These findings indicate that MPA has the potential to alter epithelial barrier integrity in women using DMPA.

Changes in the vaginal microbiome that cause infections such as bacterial vaginosis and other reproductive infections can increase genital inflammation and are pro-inflammatory risk factors for HIV acquisition (Buvé et al., 2014, Fichorova et al., 2015). Previous studies investigating
the effect of hormonal contraceptives on the vaginal microbiome have shown that women using combined oral contraceptives and women using DPMA have a decreased prevalence of bacterial vaginosis (Van de Wijgert et al., 2013, Vodstrcil et al., 2013). A recent study has also shown that altered vaginal microbiota is associated with the observed differences in the effects of hormonal contraceptives on the cervical mucosal immune environment, in HIV negative women, providing a possible reason for the differences in effects of hormonal contraceptives like MPA in different populations (Fichorova et al., 2015).

The effect of MPA on soluble immune mediators, HIV target cells and co-receptors, as well as on genital tract permeability, supports the associated risk of HIV acquisition with MPA use by providing biological evidence for possible mechanisms. Previous studies have compared MPA to other contraception forms and a few have shown findings when comparing MPA to NET or LNG, but there is an urgent need for more information about the effect of other progestins, such as ETG and NES, on soluble immune mediators in different cell types in the female genital tract, on HIV target cell frequency and activation, and on epithelial barrier integrity (Hapgood et al., 2014b). This information is important due to these progestins being considered for use intravaginally in combination with ARVs.

1.5 The role of antiretroviral drugs in the prevention of HIV/AIDS

Decades after the emergence of the epidemic, a cure for HIV/AIDS is still not available. However, effective ARVs used to treat the disease have allowed people living with the disease to lead long lives. Treatment of HIV is carried out using a combination of three or more ARV drugs referred to as highly active antiretroviral therapy (HAART) (Arts and Hazuda, 2012). ARVs substantially inhibit HIV viral production at different stages of its life cycle, allowing host immune responses to recover.

ARVs target various stages of the HIV life cycle (Figure 1.2) (Ramjee, 2011). When the virus is exposed to a host target cell, it attaches to the cell through the binding of the viral envelope (Env) protein to the primary cellular receptor for the virus, CD4, and then binds to the co-receptors, C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) (Kirchhoff, 2013). In vivo, HIV-1 predominantly binds to the CCR5 co-receptor, while some HIV variants are able to bind to the CXCR4 co-receptor. This gives rise to the phenomenon known as HIV tropism, where R5-tropic viruses bind to CCR5 expressing cells, and X4-tropic viruses bind to CXCR4 expressing cells (Symons, 2014). ARVs that target this stage of the life cycle are called co-receptor inhibitors (CRIs) and function by inhibiting the binding of CD4 or the co-receptors to the Env protein (Neves and Sarmento, 2014). After attachment, several conformational changes occur, which cause fusion of viral and cellular
membranes to form pores, and this then allows entry of viral genetic material into the cell (Kirchhoff, 2013). ARVs, which target this stage are called fusion inhibitors (FIs) (Neves and Sarmento, 2014). Examples of a CRI and a FI are maraviroc and fuzeon, respectively.

After fusion and uncoating, the viral DNA forms a viral reverse transcription complex. In this complex, single stranded viral DNA is reverse transcribed into proviral double stranded DNA by the viral enzyme reverse transcriptase (Kirchhoff, 2013). The ARVs that target viral replication at this step are known as reverse transcriptase inhibitors. Nucleotide or nucleoside reverse transcriptase inhibitors (NRTIs) are analogues of nucleotide or nucleoside monophosphates, which lack the 3’ hydroxyl group necessary for the formation of a phosphodiester linkage with the next incoming nucleoside, acting as chain terminators of the polymerisation chain reaction (Neves and Sarmento, 2014). Emtricitabine (FTC) and tenofovir (TFV) are examples of NRTIs. ARVs that directly inhibit the reverse transcriptase enzyme are known as non-nucleoside reverse transcriptase inhibitors (NNRTIs), and these act by binding to the enzyme and inducing conformational changes to the active site (Hughes, 2015). Efavirenz (EFV) and nevirapine are NNRTIs commonly used in HAART.

Proviral DNA is then synthesised and integrated into the host genome by the viral enzyme integrase. The integrated proviral DNA is transcribed into mRNA, which is then translated to produce HIV proteins that assemble into virions and bud out of the cell (Kirchhoff, 2013). For these virions to become mature and infectious, they are processed by the HIV enzyme protease. ARVs that target the viral integration and mature virion formation are integration inhibitors (INIs) and protease inhibitors (PIs) and include ARVs such as raltegravir and lopinavir (Neves and Sarmento, 2014).

There are several U.S. Food and Drug Administration (FDA) approved ARVs currently available. The World Health Organization (WHO) has recommended combinations of tenofovir disoproxil fumarate (TDF) with either lamivudine or FTC, and EFV for people starting HIV treatment (WHO, 2015). HAART has also led to the identification of ARVs that can be used for pre-exposure prophylaxis (PrEP) as topical microbicides and oral PrEP.
ARVs target various stages of the HIV life cycle. Co-receptor inhibitors (CRIs) target viral entry. Fusion inhibitors target viral-cell fusion, nucleos(t)ide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) target viral reverse transcription, integrase strand transfer inhibitors target viral integrase, and protease inhibitors inhibit viral protease processing of viral proteins. Figure adapted from (Neves and Sarmento, 2014).

Microbicides are topical products currently being developed to protect healthy people from becoming infected with HIV during sex. With women being the most vulnerable to HIV infection through heterosexual sex, intravaginal delivery of microbicides is the method of choice. Currently microbicide gels, vaginal rings and vaginal films are being investigated for delivery. Investigations to date have shown that the ARVs tenofovir, dapivirine, and maravirocc are good candidate microbicides for PrEP (Kelly, 2011).

1.6 Pre-exposure prophylaxis ARVs and their mechanism of action

1.6.1 Tenofovir and TDF

TFV is an NRTI, which is an analogue of adenosine monophosphate (AMP). After phosphorylation to its active form tenofovir diphosphate, it competes with deoxyadenosine 5’-triphosphate (dATP) for incorporation into newly forming HIV DNA (Antoniou et al., 2003). TFV has an in vitro IC$_{50}$ of 27 µM (Table 2) (Mesquita et al., 2012, Taneva et al., 2015). Drugs are delivered at much higher concentrations in tissue, and TFV concentrations of approximately 180 µM have previously been detected in cervicovaginal fluid (Karim et al., 2011).

TFV was the first ARV to be formulated as a 1% gel and used as a microbicide. It was first shown to be effective in protecting against SIV infection in non-human primates as a topical
vaginal product (Cranage M., 2008, Parikh U.M., 2009). The first clinical efficacy trial for 1% tenofovir gel was the CAPRISA 004 trial, which was a double-blind, randomized controlled trial that compared tenofovir gel with placebo gel in sexually active, HIV-uninfected 18- to 40-year-old women in KwaZulu-Natal, South Africa. The gel was applied pericoitally (before and after intercourse), and after 30 months, tenofovir gel reduced HIV acquisition by 39% overall and by 54% in women with high gel adherence. Two other clinical trials that followed to confirm the CAPRISA study were the Microbicide Trials Network (MTN 003/VOICE) study and the FACTS 001 trial, and both failed to show protection against HIV infection with the 1% tenofovir gel (CONRAD, 2011, MTN, 2011). The poor performance of these gels to protect against HIV has largely been attributed to poor adherence (Mansoor et al., 2014).

More promising results are shown for the lipophilic esterified prodrug form of tenofovir that contains fumaric acid, known as TDF. It is hydrolysed to TFV intracellularly, and phosphorylated to the active metabolite tenofovir diphosphate. TDF was designed to improve solid state stability (Fardis and Oliyai, 2007). Due to its high oral bio-availability, TDF has replaced the use of tenofovir as an HIV treatment drug (Fardis and Oliyai, 2007). TDF has also shown more potential for use as a microbicide, than tenofovir. The lipophilic nature of TDF allows it to permeate cell membranes more rapidly and this allows its rapid intracellular accumulation (Robbins et al., 1998). In a study where the mechanisms of uptake of tenofovir and TDF in human vaginal epithelial and T cells were investigated, it was shown that TDF permeates the plasma membrane by passive diffusion and hence there was a greater cellular accumulation of TDF, compared to tenofovir (Taneva et al., 2015). In vitro studies have also shown that TDF has a much lower IC₅₀, of approximately 0.09 µM compared to TFV at 27 µM (Table 2) (Mesquita et al., 2012, Taneva et al., 2015).

TDF showed successful protection against HIV infection, when administered in combination with emtricitabine (FTC) and delivered as an oral tablet. In the TDF2 study (Thigpen et al., 2012), HIV-seronegative men and women received either TDF-FTC or matching placebo once daily, and at the end of the study they found that TDF-FTC showed an overall reduction in HIV incidence of 62.2%. This oral drug was approved for use for PrEP by the FDA in 2012 and is currently being marketed as Truvada®. Truvada® is approved in African countries including Kenya and South Africa, and is currently given to uninfected individuals at high risk of acquiring HIV, including individuals with infected partners or individuals who engage in sexual activity in a high risk area (Bekker et al., 2016). TDF was shown to be safe and well-tolerated as an intravaginal ring (IVR) in a randomised placebo-controlled trial, which was conducted to assess the safety and pharmacokinetics of the TDF IVR in healthy, sexually abstinent women (Keller et al., 2016).
1.6.2 Dapivirine

Dapivirine (DPV) is a potent NNRTI, which inhibits a broad panel of HIV-1 isolates including wild type HIV-1 and a wide range of other NNRTI-resistant isolates (Fletcher et al., 2009). It has specific hydrophobic groups (Table 2) that bind to the reverse transcriptase and alter its ability to function, which in turn disrupts HIV-1 replication (D'Cruz and Uckun, 2006). DPV was first evaluated as a vaginal microbicide in humanised severe combined immunodeficient (hu-SCID) mouse models, where DPV was given as a gel formulation 20 minutes before vaginal cell-associated HIV challenge and was able to prevent systemic infection (Woolfson et al., 2006). In vitro studies have previously shown that, after pre-exposure to virus and during viral infection, the IC\textsubscript{50} of DPV was much lower in a cervical explant model (0.2 nM), compared to the PM-1 T cell line model (6 nM) (Fletcher et al., 2009).

DPV’s potent activity against HIV-1 led to its development into Phase III clinical trials, assessing its efficacy as a microbicide, delivered intravaginally in healthy HIV negative women. In the recent MTN-020–ASPIRE study, a randomised, double-blind, placebo-controlled trial of a monthly vaginal ring containing DPV, involving women between the ages of 18 and 45 years in Southern Africa, DPV reduced the incidence of HIV-1 infection by an overall 27%. The efficacy differed greatly according to age with an efficacy of 61% in women aged 25 and above, and 10% in women aged below the age of 25. Adherence was lower among women aged 18 to 21 and was much higher for those aged above 21, which led to the differences in efficacy (Baeten et al., 2016). In the IPM 027 clinical trial (The Ring Study), conducted in Uganda and South Africa, the monthly DPV vaginal ring reduced the risk of HIV infection by an overall 31% (Nel et al., 2016). The efficacy of the ring increased to 37% in women older than 21 years and this was also attributed to higher adherence in women older than 21 (Nel et al., 2016).

As a hydrophobic molecule, DPV is believed to be transported into tissue through passive diffusion. Since passive diffusion is a concentration dependent process, higher concentrations of DPV delivered topically lead to higher concentrations of DPV in tissues (Akil et al., 2014). Intravaginal concentrations of DPV are reported to range between 2 to 21 µM and these concentrations are much higher than the IC\textsubscript{50} of the drug (Parsons et al., 2014). DPV was found to be safe and well tolerated in the ASPIRE trial; however, in The Ring Study, use of the DPV ring was associated with abnormal uterine bleeding, pelvic discomfort or pain, lower abdominal pain, and pain where the ring was located (Nel et al., 2016).
1.6.3 Maraviroc

Maraviroc (MVC) is a CRI, which inhibits HIV-1 gp-120 (a glycoprotein found on the Env) from binding to CCR5 (Dorr et al., 2005). *In vitro*, MVC inhibits viral entry at an IC$_{50}$ of 11 nM (Dorr et al., 2005). MVC was the first small-molecule CCR5 inhibitor to be included in HAART. Initial studies on the efficacy of MVC in non-human primates showed that MVC formulated as a vaginal gel protected against HIV acquisition (Malcolm R.K., 2013). In a Phase I clinical trial involving 48 HIV negative U.S. women (IPM-026/MTN013), MVC was formulated as a vaginal ring in combination with DPV and this ring was found to be safe and well tolerated; although, very low levels of MVC were detected in tissue (Chen BA and Husnik M, 2015). The ring was found to be well tolerated and with good adherence (van der Straten et al., 2016).

The above-mentioned studies on the efficacy of candidate microbicides highlight the importance of adherence, to obtain greater protection against HIV infection. The cause of low adherence in blinded clinical trial settings is still unclear but may be influenced by factors such as timing, dosage and direction of use of topical microbicides (Woodsong et al., 2013). However, the evidence of greater protection with greater adherence shows the need for improved adherence strategies along with the introduction of topical microbicides for women at high risk of HIV infection. The ARVs investigated in the present study are TDF and DPV and were chosen due to their increased promise as microbicides for PrEP and their advancement into Phase III clinical trials.

1.7 Mucosal effects of topical ARVs in the female genital tract

The efficacy of topically delivered microbicides can be affected by many factors, which could favour mucosal infection. These include damage or inflammation of the protective epithelium, disturbance of the normal flora or reduction of innate protective factors in the mucosa (Haase, 2011). Epithelial cells in female genital mucosa not only provide a physical barrier for protection against pathogens but also act as key initiators of the innate and adaptive immune responses by secreting cytokines and chemokines.
Table 2: PrEP ARVs in phase II/III clinical trials

<table>
<thead>
<tr>
<th>ARV</th>
<th>Trial</th>
<th>Population</th>
<th>Adherence</th>
<th>Overall Decrease in HIV incidence</th>
<th>In vitro IC50 (nM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDF (Oral)</td>
<td>TDF2 study</td>
<td>Heterosexual men and women in Botswana</td>
<td>79%</td>
<td>0.0</td>
<td>90</td>
<td>Thigpen et al. 2012* Mesquita et al. 2012*</td>
</tr>
<tr>
<td>TFV (1% microbicidal gel)</td>
<td>CAPRISA 004 study</td>
<td>Women in South Africa</td>
<td>61%</td>
<td>39%</td>
<td>27000</td>
<td>Abdool Karim et al. 2010* Mesquita et al. 2012 Taneva et al. 2015</td>
</tr>
<tr>
<td>DPV (Intravaginal Ring)</td>
<td>ASPIRE study</td>
<td>Women in South Africa, Malawi, Uganda, Zimbabwe</td>
<td>70%</td>
<td>37% (excl. low adherence sites)</td>
<td>0.2-6</td>
<td>Baeten et al. 2016* Fletcher et al. 2009*</td>
</tr>
</tbody>
</table>

Notes: * References for adherence and efficacy of ARVs. * References for in vitro IC50

These cytokines and chemokines can provide an activated immune state that can fight against pathogens, or can provide a suitable environment for pathogens like HIV to enter, due to increased numbers of HIV target cells in this area (Wira et al., 2014). It is necessary for microbicides to be evaluated for these factors and other off-target effects, which may result in adverse side effects and loss of efficacy.

In a study by Hladik et al., a 1% tenofovir gel was assessed by genome-wide microarray analysis on 9 cm rectal biopsies and tenofovir, at concentrations of 50 and 500 µM, was also assessed in vitro on primary vaginal epithelial cells from four healthy women (Hladik et al., 2015). In the biopsies the 1% tenofovir gel was found to affect a much broader range of genes compared to the control nonoxynol-9 (N-9) 2% gel and hydroxyethyl cellulose (HEC) placebo gel. These included genes involved in keratinocyte differentiation and cellular innate immunity, and induced genes involved in DNA damage repair. Tenofovir suppressed anti-inflammatory factors such as IL-10 and genes important in biological processes involving the anti-inflammatory mediator TGF-β. Tenofovir also induced several chemokines including CCL19, and CCL21, which are ligands for T cells and dendritic cells. Higher densities of CD3+ and CD7+ T cells were also observed after seven days of tenofovir gel use in rectal biopsies. Similar effects of tenofovir were observed in primary vaginal epithelial cells. This study suggested that tenofovir causes a state of potential hyper-responsiveness to external
inflammatory signals but may not be directly causing inflammation (Hladik et al., 2015). In another study, tenofovir was found to directly modulate pro-inflammatory cytokines and nucleotidases in the female genital tract (Biswas et al., 2014). In this study, tenofovir was shown to enhance IL-8 and TNF-α secretion by primary epithelial cells in the endometrium and ectocervix, but not in the endocervix. Nucleotidase biological activities were significantly decreased by tenofovir in epithelial and CD4+ T cells in the endocervix and endometrium, but increased in fibroblasts in the endometrium.

Pre-clinical evaluation of DPV in cell line and ex-vivo models has shown that DPV has a low inflammatory potential, by not inducing significant changes in the release of pro-inflammatory cytokines and chemokines in the female genital tract (das Neves et al., 2016, Gali et al., 2010). DPV has also been shown to change the expression of different drug transporters in the female genital tract, which may influence the uptake of DPV as well as other ARVs or hormonal contraceptives it may be combined with (Hijazi et al., 2015). The mucosal effects of MVC are still not well reported as it is still under development as a microbicide product. The increased risk of HIV is associated with increased inflammation (Masson et al., 2015); thus, it is important to investigate the effect of ARVs on the local mucosal immune environment. The ability of ARVs like tenofovir to modulate the immune environment in the vaginal mucosal area, shows the potential of microbicides to affect immune-related factors.

1.8 Interaction between ARVs and hormonal contraceptives

The emergence of long acting microbicide ARVs delivered in vaginal rings has prompted the investigation of delivering a contraceptive along with the ARV in a combination vaginal ring MPT product. Current vaginal combination rings in Phase I clinical trials include those releasing the hormonal contraceptive, LNG, along with the anti-retrovirals, TFV or DPV. LNG was chosen as an ideal contraceptive candidate as it is already used as an IUD, and has been shown to be safe and effective at providing contraceptive efficacy when released at rates of 20 – 30 µg per day, intravaginally (Nilsson et al. 1982). Concentrations of LNG in the endometrial tissue can reach an average of 2.5 µM (Nilsson et al. 1982) which is a concentration considerably higher than the serum concentrations (0.3 – 28 nM) (Nilsson et al. 1982). Other intravaginal hormonal contraceptives that are also candidates for MPTs are ETG and NES. As these combination vaginal rings are still in early Phase I clinical trials, there is currently no data available showing concentrations of each drug needed at the female genital tract to effectively prevent HIV and pregnancy in women. However limited data on ARV intravaginal concentrations and data on intravaginal contraceptives shows that much higher concentrations of the drugs are found in tissue (Table 3).
Table 3: Intravaginal concentrations of ARV and progestin candidates for MPTs

<table>
<thead>
<tr>
<th>Product</th>
<th>Concentration released in product</th>
<th>Cervicovaginal fluid concentration (µM)</th>
<th>Cervical tissue concentration (µM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravaginal microbicide ARVs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFV gel</td>
<td>1 % (v/v) gel*</td>
<td>41 – 34 000</td>
<td>0.7 – 4 800</td>
<td>Schwartz et al. 2011, Karim et al. 2011</td>
</tr>
<tr>
<td>DPV ring</td>
<td>25 mg*</td>
<td>17</td>
<td>2 - 21</td>
<td>Chen et al. 2015, Nel et al. 2010</td>
</tr>
<tr>
<td>Intravaginal progestins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETG/EE ring (NuvaRing®)</td>
<td>120 µg ETG** 15 µg EE</td>
<td>Not determined</td>
<td>0.002*</td>
<td>Roumen et al. 2006</td>
</tr>
<tr>
<td>LNG-IUD</td>
<td>30 µg**</td>
<td>Not determined</td>
<td>2.5*</td>
<td>Nilsson et al. 1982</td>
</tr>
<tr>
<td>NES/EE ring</td>
<td>150 µg NES** 15 µg EE</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Sivin et al. 2005</td>
</tr>
<tr>
<td>MPT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPV-LNG ring</td>
<td>200 mg DPV* 32 mg LNG</td>
<td>Not determined</td>
<td>Not determined</td>
<td>IPM</td>
</tr>
<tr>
<td>TFV-LNG ring</td>
<td>8 – 10 mg TFV** 20 µg LNG</td>
<td>Not determined</td>
<td>Not determined</td>
<td>CONRAD</td>
</tr>
</tbody>
</table>

Notes: * - pericoitally applied, * - single dose, ** - daily dose, + - average tissue concentration

The potential of high intravaginal concentrations of ARVs and high contraceptive levels to alter steroid receptor activity or to modulate expression of immune function genes via steroid receptor mediated mechanisms is also an important consideration for the development of combination vaginal MPTs. A computational docking study showed that a panel of ARVs including maraviroc can bind to liver X receptors (α and β), which are part of the nuclear receptor family (Svard et al., 2014). This suggests that microbicide ARVs may have the potential to interact with other nuclear receptors, such as the PR, the GR or the AR in the female genital tract and this may result in off-target side effects of ARVs.

In a recent study, presented at the HIV Research for Prevention conference (HIVR4P) 2016, the interaction between DPV and LNG, when delivered together in a vaginal ring, was investigated in vitro. In this study the authors investigated whether DPV would impede the action of LNG, using binding assays of DPV to the PR; and they investigated whether LNG would affect the action of DPV using HIV inhibition assays in TZM-bl and PM-1 cells. Data from the study revealed that there was low potential for DPV to impede the pharmacodynamic action of LNG and that there was no indication that LNG would inhibit the pharmacodynamic activity of DPV in vitro (Holt J et al., 2016). In clinical studies, DMPA’s contraceptive efficacy does not appear to be reduced by ARVs, such as EFV and nevirapine (Nanda et al., 2008),
and it was shown not to reduce the prophylactic effect of FTC and TDF in macaques (Radzio et al., 2014).

Although a few pharmacodynamic studies have investigated the interaction between ARVs and progestins, there is no information available on the effects of ARVs in combination with progestins on immunomodulatory gene expression and steroid receptor function. Combinatory effects may have implications for the risk of HIV acquisition as well as steroid receptor mediated side effects.

1.9 Significance, hypothesis and aims

With the increasing development of MPTs that deliver an ARV and a hormonal contraceptive in a single product, the interaction between these two compounds in the female genital tract needs to be considered. There is a need to understand how these compounds may influence expression of immunomodulatory genes, which may affect HIV acquisition in the female genital tract, independently and in combination with each other. It is also important to consider steroid receptor involvement in the cells of the female genital tract. Steroid receptors are involved in various physiological functions and any effects of ARVs alone or in combination with progestins on steroid receptor activity may contribute to off-target side effects in women taking hormonal contraceptives and ARVs simultaneously.

The key immunomodulatory genes that were chosen for investigation in the present study are the pro-inflammatory interleukins, IL-8 and IL-6, as well as the anti-inflammatory glucocorticoid induced leucine zipper (GILZ). IL-6 is an important cytokine that is involved in regulating T cell activation, differentiation and growth, as well as cytotoxic immunity against virus infected cells (Borish and Steinke, 2003). A previous study showed that decreased levels of IL-6 were associated with HIV acquisition (Lehman et al., 2014). IL-8 is a chemokine that is involved in recruitment of neutrophils and T cells to sites of infection and has previously been shown to increase susceptibility to HIV-1 in cervical tissue explants (Borish and Steinke, 2003, Narimatsu et al., 2005). GILZ is expressed constitutively in various cell types including epithelial cells, macrophages and dendritic cells (Ayroldi and Riccardi, 2009). It is strongly induced by glucocorticoids and mediates the anti-inflammatory effect of glucocorticoid driven responses (Ayroldi and Riccardi, 2009). These genes were ideal to investigate because of their important roles in mediating immune responses related to HIV acquisition, as well as their ability to be regulated by glucocorticoids and the hormonal contraceptive MPA via the GR (Govender et al., 2014, Verhoog et al., 2011).
TZM-bl cells are epithelial cervical cells, that are a good indicator cell line for HIV infection. They are HeLa cells engineered to constitutively express the HIV co-receptors, CD4 and CCR5 (Platt et al., 1998). Cervical epithelial cells have also been shown to express inflammatory cytokines (Fichorova and Anderson, 1999). Thus, these cells were a good model to investigate immunomodulatory gene regulation. Cervical tissue explants were chosen as a good model to provide the physiological relevance of the study as this would represent the effects that could happen in vivo.

As depicted in Table 3, physiologically relevant intravaginal tissue concentrations of TDF (which releases active TFV-DP) and DPV range from 2 - 30 µM (Chen BA and Husnik M 2015; Keller et al. 2016; Nel et al. 2010). Thus, in the current study it was necessary to investigate concentrations that mimic those found in vivo, that would be non-toxic to the cells. A dose response was performed and we chose the highest possible concentration that would not be toxic to the cells. Similar to the ARVs, progestin concentrations in the female genital tract often differ to serum concentrations, and some have been shown to be much higher in vaginal tissue (Miles et al. 1994; Nilsson et al. 1982). There are currently very limited data on the concentration of different progestins in female genital tract tissues, with data available focusing mainly on IUDs and vaginal rings. In the present study, the progestin concentration chosen was 100 nM and this concentration was suitable as it is higher than the average peak serum concentrations, yet also within the range of potencies able to elicit an immune response in cervical cells as seen from previous studies with MPA (Govender et al., 2014, Koubovec et al., 2005, Ronacher et al., 2009).

**Hypotheses**

The first hypothesis of the study is that ARVs on their own and in combination with progestins may have differential effects on the expression of immune function genes in cells of the female genital tract. This may occur through altering steroid receptor levels or steroid receptor activation in the female genital tract. The second hypothesis is that the progestin MPA alone and in combination with ARVs will directly affect HIV replication and alter HIV co-receptor expression in cervical cells and tissues.

**Aims**

The specific aims of this study are to:

1.) Investigate whether the ARVs DPV and TDF, and a panel of progestins, alone or in combination with each other, differentially affect expression of steroid receptor regulated genes involved in HIV-1 acquisition in TZM-bl cervical cells and ectocervical
tissue explants. The selected target genes to be investigated include IL-8, IL-6 and GILZ.

2.) Investigate the role of ARVs and progestins alone and in combination on endogenous steroid receptor levels in TZM-bl cells, and the role of ARVs on steroid receptor transcriptional activity.

3.) Investigate the effect of MPA in combination with ARVs on HIV replication and HIV coreceptor expression.

This study will provide insight into the choice of progestin/ARV combinations that will minimise side effects and will be safe for use in women at risk of HIV-1 infection.
Chapter 2: Materials and methods

2.1 Test compounds and antibodies

The following compounds were used for the study: (11b,16a)-9-fluoro-16-methylpregna-1,4-diene-3,20-dione (dexamethasone; DEX), 6α-methyl-17α-hydroxyprogesterone acetate (medroxyprogesterone acetate; MPA), 17α-ethynyl-19-nortestosterone (norethisterone; NET), 13β-ethyl-17α-ethynyl-17β-hydroxy-4-en-3-one (levonorgestrel; LNG), 16-methylene-17α-acetoxy-19-norpregn-4-ene-3,20-dione (nestorone; NES), (17α)-13-ethyl-17-hydroxy-11-methylene-18,19-dinorpregn-4-en-20-yn-3-one (etonogestrel; ETG), and 7α,17α-dimethylestr-4-en-17β-ol-3-one (mibolerone; MIB). The steroids were obtained from Sigma-Aldrich, except for MIB, which was obtained from Perkin Elmer. These ligands were all made up to a stock concentration of 10^{-2} M in absolute ethanol (EtOH; Merck, South Africa).

The ARVs [[[2R]-1-(6-aminopurin-9-yl) propan-2-yl]oxymethyl-(propan-2-yloxy)carbonyloxy-methoxy]phosphoryl]oxymethyl propan-2-yl carbonate; (E)-but-2-enedioic acid (tenofovir disoproxil fumarate; TDF) and 4-[[4-(2,4,6-trimethylanilino)pyrimidin-2-yl]amino]benzonitrile (Dapivirine; DPV) were both purchased from Selleck Chemicals (USA). The ARVs were made up to a stock concentration of 10^{-2} M in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Stock concentrations of ARVs were serially diluted by 1:10 with DMSO for dose response experiments, from 10^{-2} M to 10^{-6} M. Stock concentrations of steroids were also serially diluted by 1:10 with EtOH to concentrations ranging from 10^{-2} M to 10^{-6} M. All test compounds (ARVs, progestins, steroid receptor ligands) were added to cells at a final concentration of 0.1% (v/v), unless otherwise stated.

The following primary antibodies were obtained from Santa Cruz Biotechnology (Germany), and used for the study: GR (H-300; sc-8992), PR (C-20; sc-539), AR (441; sc-7305), and GAPDH (0411; sc-47724). Antibodies were all tested prior to this study and were established to have no cross reactivity with each other (data not shown). Secondary antibodies were also obtained from Santa Cruz Biotechnology, and the following were used: anti-mouse (sc-2005) and anti-rabbit (sc-2313).

2.2 Plasmids

The human GR plasmid, pcDNA3-hGR was a gift from Prof. D. W. Ray (University of Manchester, UK) (Ray et al., 1999). The human AR plasmid, pSVARo (Brinkmann et al., 1999) was obtained from Frank Claessens (University of Leuven, Leuven, Belgium). The pTAT.GRE-E1b-luc (pTAT-GRE) luciferase reporter gene plasmid, which contains two glucocorticoid response elements (GRE) from the rat tyrosine aminotransferase (TAT) gene, was a gift from...
Dr. G. Jenster (Erasmus University of Rotterdam, Rotterdam, Netherlands) (Sui et al., 1999). The pcDNA3 plasmid (empty vector), which lacks an inserted downstream DNA sequence, was obtained from Invitrogen, UK. An R5-tropic infectious molecular clone, HIV-1_{BaL}, that has a luciferase gene inserted adjacent to the env gene in the HIV-1 NL4-3 backbone known as NL–LucR.T2A BaL.ecto, was a gift from Dr. Christina Ochsenbauer (Edmonds et al., 2010). In the present study, the clone was named HIV-1_{BaL Renilla}.

2.3 Plasmid transformation and preparation

Plasmid transformation was performed according to Sambrook and Russell (Sambrook and Russell, 2006). Steroid receptor plasmids (10 ng) were transformed into 100 µl competent _Escherichia coli_ DH5α cells and incubated for 30 minutes on ice after which heat shock at 42°C for 90 seconds was performed and cells were returned to ice for two minutes. Thereafter 900 µL of Super Optimal broth with Catabolite repression (SOC) media was added (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5mM KCl, 10 mM MgCl₂ and 20 mM glucose) and the cell mixture was incubated at 37°C in a shaking incubator for 1 hour at 200 rpm. Cell culture was then diluted with SOC media and spread plated onto Luria broth (LB) agar plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl and 1.5% (w/v) agar with 100 µg/ml ampicillin) with the appropriate controls and incubated overnight at 37°C. The following day, 5 mL LB starter cultures supplemented with ampicillin (100 µg/mL) were inoculated with single colonies for 8 hours at 37°C, while shaking. Thereafter, 200 µL of starter culture was inoculated into 200 mL LB supplemented with ampicillin (100 µg/mL), and incubated overnight at 37°C while shaking. Plasmids were then purified using the NucleoBond® Xtra Maxi plasmid DNA purification kit (Macherey-Nagel) according to the manufacturer’s instructions. Plasmid DNA concentration and purity was assessed with a NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies).

2.4 Restriction enzyme digest

Confirmation of plasmid identity was assessed by restriction enzyme digest. 500 ng of plasmid DNA was digested using the FastDigest™ restriction enzymes (Fermentas, Thermo Scientific, USA) for 10 minutes with a corresponding undigested control. DNA was electrophoresed on a 0.8%, 1 X TRIS acetate EDTA (TAE) agarose gel with 10 µg/ml ethidium bromide (Sigma-Aldrich, South Africa) for 1 hour at 60 V. The gel was visualised on a Syngene, G:Box (Vacutec, England) and images were acquired using GeneSnap from synGene, version 7.08 (SynGene, England).
2.5 Cell culture

The human cervical TZM-bl cell line was obtained from the NIH AIDS Reagent Program. TZM-bl cells are a HeLa cell line stably transfected with the HIV receptor CD4 and co-receptor, CCR5. The cells contain copies of the luciferase and β-galactosidase genes under the control of the HIV-1 promoter (LTR). U2OS cells are a human osteosarcoma cell line that were purchased from America Type Culture Collection (ATCC, USA). COS-1 (African green monkey kidney fibroblast) cells, used in the present study to generate western blot positive controls, were also purchased from ATCC. HEK293T cells, a human embryonic kidney cell line, were used in the present study to generate infectious molecular clones and were obtained from ATCC. Cells were grown in 75 cm² flasks in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 1 mM sodium pyruvate (58636, Sigma-Aldrich, South Africa), 44 mM sodium bicarbonate (Sigma-Aldrich, South Africa), 10% (v/v) foetal bovine serum (Thermo Scientific, South Africa), 100 IU/mL penicillin, and 100 mg/mL streptomycin (P4333, Sigma-Aldrich, South Africa). Cells were maintained at 37°C in a water jacket incubator (90% humidity and 5% CO₂). Cells were passaged twice a week, with 0.25% (w/v) trypsin/0.1% (w/v) EDTA in phosphate buffered saline (PBS, Highveld Biological, South Africa). Mycoplasma-negative cells were used in all experiments and cells were routinely tested for mycoplasma infection by means of Hoechst staining (Chen, 1977).

2.6 TZM-bl cell stimulation and harvesting

2.6.1 Progestin treatment of TZM-bl cells

TZM-bl cells were seeded at a density of 1 x 10⁵ cells/well in 12-well flat-bottom plates and incubated at 37°C for 24 hours. Cells were then treated with 100 nM DEX, MPA, NET, LNG, NES and ETG. EtOH (0.1% v/v) was used as the vehicle control and the cells were then incubated at 37°C for 24 hours. Cells were washed with PBS and harvested for total RNA with 400 µL of TRIzol® (Sigma Aldrich, South Africa). Western blot samples were harvested using 50 µL of 2 X SDS sample buffer (100mM Tris pH 6.8, 5% v/v SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol, 0.1% w/v bromophenol blue) then boiled at 100°C and stored at -20°C.

2.6.2 ARV dose response experiments

TZM-bl cells were seeded at a density of 1 x 10⁵ cells/well in 12-well flat bottom plates and incubated at 37°C for 24 hours. Cells were then treated with varying concentrations of ARVs ranging from 1 nM to 1 µM and dimethyl sulfoxide (DMSO, 0.1% v/v) was used as the vehicle control. Cells were then incubated at 37°C for 24 hours. Thereafter cells were harvested for total RNA with 400 µL of TRIzol®.
2.6.3 ARV and progestin combination treatment

TZM-bl cells were seeded at a density of $1 \times 10^5$ cells/well in 12-well flat bottom plates and incubated at 37°C for 24 hours. Cells were then treated with 100 nM DEX and MPA, and 1 µM TDF and DPV. Cells were also treated with combinations of these (DEX and TDF, DEX and DPV; MPA and TDF, MPA and DPV). EtOH (0.1% v/v) and DMSO (0.1% v/v) were used as vehicle controls and the cells were then incubated for 24 hours. Supernatants were harvested for ELISA assays and stored at -80°C. Cells were then harvested for total RNA with 400 µL of TRIzol®. Western blot samples were harvested using 50 µL of 2 X sample buffer (100mM Tris pH 6.8, 5% v/v SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol, 0.1% w/v bromophenol blue) then boiled at 100°C for 10 minutes and stored at -20°C.

2.6.4 Cell viability assay

Cell viability assays were performed with 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole that is reduced to purple formazan in living cells, according to Edmondson et al. (Edmondson et al., 1988) using the In Vitro Toxicology Assay Kit, MTT based (Sigma Aldrich, South Africa). The assay was done in parallel with ARV dose response and ARV-progestin combination experiments. TZM-bl cells were seeded into 96-well flat bottom plates at a density of $1 \times 10^4$ cells/well and incubated at 37°C for 24 hours. Thereafter cells were treated with the different ligands that each experiment required and were then incubated for 24 hours. 5 mg/mL MTT reagent (Sigma Aldrich, South Africa) was prepared using PBS and filtered in a sterile laminar flow hood. MTT was added to the cells at a final concentration of 0.5 mg/mL and incubated for 2 hours. Cells were then solubilized by adding solubilisation buffer (0.1 N HCl in isopropanol) for 10 minutes. The plate was read on a spectrophotometer (Thermo Scientific, USA) at 595 nm. Cellular viability was determined by normalising each sample absorbance reading to the average vehicle control absorbance reading (Edmondson et al., 1988).

2.7 RNA isolation, cDNA synthesis and qRT-PCR

2.7.1 RNA isolation and cDNA synthesis

TZM-bl cells in TRIzol® were incubated at room temperature for 5 minutes, then transferred to 1.5 mL Eppendorf tubes. Chloroform was then added to each tube, vortexed for 30 seconds and allowed to stand for 3 minutes. Samples were then centrifuged at 20 000 x g at 4°C for 15 minutes. The aqueous phase was then transferred into a new Eppendorf tube and 200 µL of isopropanol was added to the samples and mixed by inversion. Samples were then incubated at room temperature for 10 minutes. Samples were subsequently centrifuged at 20
000 x g for 10 minutes at 4°C to pellet the RNA. The supernatant was removed, and the pellet was washed twice in 70% EtOH (in DEPC-treated H2O). Samples were centrifuged at 20 000 x g for 5 minutes at 4°C and the supernatant was carefully removed to ensure that all EtOH had been removed. Samples were then air dried for 5 minutes and the pellets were resuspended in 10 µL RNase-free DEPC treated H2O.

RNA quantity and quality was assessed using the Nanodrop spectrophotometer (Thermo Scientific). Only good quality RNA with 260/280 ratios >1.8 and 260/230 ratios between 2 and 2.2 was used. RNA integrity was assessed by running 250 ng of RNA on a denaturing formaldehyde agarose gel in MOPS buffer (0.2 M MOPS in DEPC water, 0.05 M CH3COONa and 0.01 M EDTA) to detect 18S and 28S ribosomal RNA bands. 3.3 µL RNA loading buffer (12% (v/v) DEPC water, 5% (v/v) bromophenol blue solution, 7% (v/v) glycerol, 10% (v/v) 10 X MOPS, 17% (v/v) 12.3 M formaldehyde and 49% (v/v) formamide) was added to the RNA samples and these were analysed by gel electrophoresis on a 1% agarose formaldehyde gel (70% (v/v) DEPC water, 10% (v/v) 10 X MOPS buffer and 20% (v/v) formaldehyde) for 25 minutes at 65 V in 1 X MOPS buffer. Bands were visualised on a SynGene, G:Box (Vacutec, England) and the images were acquired using GeneSnap from SynGene, version 7.08 (SynGene, England). 250 ng RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, South Africa), according to the manufacturer's instructions. RNA was reverse transcribed by random priming with the use of a MultiScribe™ reverse transcriptase and deoxynucleotide triphosphates (dNTPs) in a 10 µL reaction for 1 hour at 37°C. The reaction was stopped by incubating cDNA at 85°C for 5 minutes and thereafter the samples were stored at -20°C until use in subsequent real time qPCR reactions.

2.7.2 qRT-PCR

Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using the FastStart Essential DNA Green Master kit (Roche) on a RotorGene 3000 (Qiagen, Netherlands) real time qRT-PCR machine, according to manufacturer’s instructions. In each 20 µL reaction, forward and reverse primers, 10 µL FastStart, RNase free H2O and 1 µL template cDNA were added. The genes investigated were GILZ, IL-8, IL-6, CCR5 and CXCR4. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The IL-8, CCR5, CXCR4 and GAPDH primers were added at a concentration of 500 nM. IL-6, was added at 250 nM, and GILZ, which is a validated primer set purchased from Qiagen South Africa, was added at a final concentration of 1 X. Forward and reverse primer sequences for each gene run are shown in Table 4. The qRT-PCR profiles for IL-6, IL-8 and GAPDH were established by Verhoog et al. (Verhoog et al., 2011) while those for CCR5 and CXCR4 were optimized for the current study and were run with an annealing temperature of 55°C and 60°C.
respectively. Each qRT-PCR profile contained a standard initial denaturation at 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds, annealing for 10 seconds and 72°C for 10 seconds, with a final elongation step at 72°C. Melting curve analysis and gel electrophoresis was performed to confirm the generated product in each sample. Standard curves were used to determine the efficiency of each primer set and relative mRNA levels were normalized to relative GAPDH transcript levels to obtain fold induction, according to the method described by Pfaffl (Pfaffl, 2001).

Table 4: Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’ – 3’)</th>
<th>Strand</th>
<th>Annealing temp (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>TCTCCACAAGCGCCCTCG</td>
<td>Forward</td>
<td>60</td>
<td>(Wolf et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>CTCAGGGCTGAGATGCCG</td>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GILZ</td>
<td>Quantitect Primer QT00091035</td>
<td>Forward and Reverse</td>
<td>60</td>
<td>(Qiagen, South Africa)</td>
</tr>
<tr>
<td>CCR5</td>
<td>TGGACCAAGCTAGCCAGGTG</td>
<td>Forward</td>
<td>55</td>
<td>(Ray et al., 2017 submitted)</td>
</tr>
<tr>
<td></td>
<td>CAGTCACAAAGCCAGCATG</td>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR4</td>
<td>GAAATGGGCTCAGGGGACTAT</td>
<td>Forward</td>
<td>60</td>
<td>(Ray et al., 2017 submitted)</td>
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<tr>
<td></td>
<td>TTCAGCCAACAGCTCTCTTG</td>
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</tr>
<tr>
<td>IL-8</td>
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<td>60</td>
<td>(Wolf et al., 2002)</td>
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<tr>
<td></td>
<td>TGCCAAGGAGTGTCAAAAG</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>TGAACGGGAAGCTACTGG</td>
<td>Forward</td>
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<td>(Ishibashi et al., 2003)</td>
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<tr>
<td></td>
<td>TGTCAAGTGATAAACCGCTGCC</td>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.8 ELISA assay

Supernatants from TZM-bl cells and cervical explants treated with ARV-progestin combinations were thawed from -80 °C. Cytokine protein levels for IL-8 and IL-6 were measured using the R&D Systems DuoSet® ELISA kit (R&D Systems, USA) according to the manufacturer’s instructions. Relative fold change expression was determined by setting the vehicle control (EtOH and DMSO) to 1.

2.9 Reporter assay

Transactivation reporter assays were optimised in U2OS cells using the method described by Ronacher et al. for the GR (Ronacher et al., 2009). U2OS cells were seeded into 10 cm plates at a density of 1.5 x 10⁶/ml and incubated for 24 hours. Thereafter, cells were transiently transfected using XtremeGene 9 (Roche Applied Science, South Africa) for 24 hours with 0.94 µg TAT-GRE, 2.5 µg pcDNA-3 (empty vector) or 2.5 µg pSV-hAR for the AR and 1.88 µg TAT-
GRE, 5 µg pcDNA-3 (empty vector) or 5 µg pcDNA3 GR WT for the GR. Cells were then replated into 96-well plates at a density of 1 x 10^4 and incubated for 24 hours. On day three, cells were washed once with PBS and were treated with the respective compounds or vehicle (EtOH and DMSO) in serum-free DMEM for 24 hours. The cells were harvested by washing twice with PBS and lysed in 50 µL 1 X Reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity in the lysates was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). The luciferase values obtained were normalized to protein content per well as determined by a Bradford assay (Bradford, 1976).

2.10 Western blotting

2.10.1 Preparation of positive controls

COS-1 cells were seeded into a 12-well plate at a density of 1 x 10^5 cells/well and incubated at 37°C for 24 hours. The cells were transiently transfected with 1 µg/well steroid receptor expression vectors (AR, GR, PR) using XtremeGene 9 and incubated for 24 hours. Cells were then harvested in 50 µL of 2 X protein sample buffer, and incubated at 100°C for 7 minutes. Samples were stored at -20°C until use.

2.10.2 Western blot analysis and quantification

Western blot analysis was performed according to Avenant et al. (Avenant et al., 2010a). Equal volumes of sample were loaded on an 8% or 10% SDS polyacrylamide gel and separated with 1 X running buffer [25 mM TRIS-HCl, 250 mM glycine and 0.1% (v/v) SDS, pH 8.4 (Sambrook and Russell, 2006)], using a Bio-Rad Mini Protean II electrophoresis set (Bio-Rad, South Africa). The protein molecular weights were estimated using the Pageruler™ Prestained protein ladder SM0671 (Fermentas Life Sciences). The separated proteins were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham, South Africa) using the Mini Protean III blotting system (Bio-Rad, South Africa) for 1 hour at 4°C in transfer buffer (25 mM TRIS, 200 mM glycine, 10% (v/v) methanol) at 180 milliamps. Membranes were blocked in 4% ECL blocking buffer which contained 4% (w/v) ECL blocking powder (Amersham, South Africa) in 1 X TRIS-buffered saline (50 mM TRIS, 150 mM NaCl; TBS) with 0.1% (v/v) Tween for 1 hour at room temperature, followed by incubation with primary antibody in 4% ECL in 1 X TBS-Tween overnight at 4°C. Primary antibodies were added in the following dilutions: AR 1:1 000, GR 1:4 000, PR 1:1 000, GAPDH 1:15 000. Membranes were then washed twice with 1 X TBS-Tween for 5 minutes and once for 10 minutes, followed by incubation with the appropriate secondary antibody in 5% (w/v) skim milk powder in 1 X TBS-Tween at room
temperature for 1 hour. The anti-rabbit secondary antibody was used for the GR and AR antibodies, and was added at a 1:10 000 dilution, while anti-mouse was used for the PR and GAPDH antibodies and was added at a 1:5 000 dilution. Thereafter the membranes were washed as before with 1 X TBS-Tween and subsequently incubated in ECL-chemiluminescent substrate (Thermo Scientific, USA) for 1 minute before being visualised using autoradiography, with varying exposure times. The proteins were visualized on Hyperfilm MP high performance autoradiography film (Amersham, South Africa). Bands on the film were scanned and quantification was done using ImageJ software (NIH, USA).

For detection of different proteins on the same membrane, membranes were stripped in stripping buffer [100 mM β-mercaptoethanol, 2% (v/v) SDS, 62.5 mM TRIS-Cl, pH 6.7, (Sambrook and Russell, 2006)] for 30 min at 65°C. Thereafter membranes were washed twice with 1 X TBS-Tween for 5 minutes and once for 10 minutes, then blocked in 4% ECL blocking buffer for 1 hour at room temperature. Antibody incubations were then performed as described above.

2.11 HIV-1 infection assay

2.11.1 Virus propagation

HIV-1\textsubscript{Bal\_Renilla} infectious molecular clones were prepared according to Pear \textit{et al.} (Pear \textit{et al.}, 1993) in HEK293T cells. HEK293T cells were plated in 10 cm plates at a density of 4 x 10\textsuperscript{6} for 24 hours. Thereafter cells were transfected using XtremeGene HP with 20 µg HIV-1\textsubscript{Bal\_Renilla} plasmid, the control plate was transfected without any plasmid and cells were then incubated for 72 hours. Supernatants were harvested into cryovials and 125 µL of charcoal stripped fetal calf serum (CS-FCS, Thermo Scientific, USA) was added to 1 mL of supernatant. To determine the 50% tissue culture infective dose (TCID\textsubscript{50}), the virus was titrated in TZM-bl cells as described by Edmonds \textit{et al.} (Edmonds \textit{et al.}, 2010). TZM-bl cells were seeded into 96-well plates at a density of 1 x 10\textsuperscript{4} cells/well and incubated for 24 hours. Cells were infected with a 5-fold dilution series of the virus in the media of the cells and incubated for 72 hours. Cells were lysed with 1 X Bright-Glo\textsuperscript{™} and the luciferase activity was measured using a luminometer (Microplate Modulus, Promega, USA). The titre of the virus stock was determined using the Reed Muench method and expressed as log infectious units (IU/ml) (Reed and Muench, 1938).

2.11.2 TZM-bl infection: Virus dose

TZM-bl cells were seeded into 96-well plates at a density of 1 x 10\textsuperscript{4} cells/well and incubated for 24 hours. Thereafter cells were treated with 100 nM DEX and MPA with EtOH (0.1% v/v)
as a vehicle control for 2 hours. Cells were then infected with the following amounts of infectious units of HIV-1_{Bal_Renilla}: 5, 10, 20, 40, 100, and 200 infectious units/well or virus control in a final volume of 100 µL for each well, and were incubated for 48 hours. Thereafter, the cells were lysed with 1 X Bright-Glo™ and the luciferase activity was measured using a luminometer (Microplate Modulus, Promega, USA). In addition, a parallel experiment was done and MTT activity was measured. Luciferase activity was normalised to MTT activity.

### 2.11.3 TZM-bl infection: ARV and progestin treatment

TZM-bl cells were seeded into 96-well plates at a density of 1 x 10^4 cells/well and incubated for 24 hours. Thereafter cells were treated with 100 nM DEX and MPA, and 1 µM TDF and DPV. Cells were also treated with combinations of these (DEX and TDF, DEX and DPV; MPA and TDF, MPA and DPV). EtOH (0.1% v/v) and DMSO (0.1% v/v) were used as vehicle controls and cells were incubated at 37°C for 24 hours. Cells were then infected with 40 infectious units/well of HIV-1_{Bal_Renilla} or virus control in a final volume of 100 µL for each well, and were incubated for 48 hours. Thereafter, the cells were lysed with 1 X Bright-Glo™ and the luciferase activity was measured using a luminometer (Microplate Modulus, Promega, USA). In addition, a parallel experiment was done and MTT activity was measured. Luciferase activity was normalised to MTT activity.

### 2.12 Explant tissue experiments

Cervical tissue was obtained from HIV-1 negative, pre-menopausal women, with a normal pap smear and undergoing hysterectomies for benign reasons, after informed consent. Ethics was obtained from the Human Research Ethics Committee (The University of Cape Town, South Africa) for the duration of this study (HREC 258/2017) Fresh tissue was supplied from two sites in the Western Cape, South Africa; namely, Groote Schuur Hospital and Tygerberg Hospital. Inclusion criteria and donor consent were confirmed prior to the operation. Blood samples were collected and sent to the National Laboratory Health Services (NHLS, Groote Schuur Hospital, South Africa) for serum antibody testing of HIV-1 and HSV 1/2 status. The majority of the samples were positive for HSV-1 and negative for HSV-2. Additionally, serum levels of luteinising hormone (LH), follicle stimulating hormone (FSH), P₄ and E₂ were evaluated and used to define the phase of the menstrual cycle. Menstrual cycle was determined using the guidelines given by the NHLS (South Africa). All donors used in this study were pre-menopausal and were not on any hormonal contraceptives.

Cervical tissue was processed as previously described by Fletcher et al. (Fletcher et al., 2005), between 1 to 3 hours post-operation. Excess underlying stromal tissue was removed from the
epithelial layer of the ectocervical tissue. The epithelial layer was then diced into 3 mm³ explant pieces which were randomly placed into separate wells of 96-well round bottomed plates. Non-polarised explants were cultured in 200 μL RPMI (Lonza, Switzerland) supplemented with 10% (v/v) CS-FCS (Thermo Scientific, USA), 2 mM L-glutamine (Sigma-Aldrich, South Africa), 10 μg/mL Fungizone (Sigma-Aldrich, South Africa), 10 U/mL IL-2, 100 IU/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, South Africa). Cervical tissue explants were stimulated in triplicate or quadruplicate with steroid ligands and ARVs in RPMI and incubated at 37°C in a water jacket incubator (90% humidity and 5% CO₂) for 48 hours. The MTT assay was performed on the tissue as a qualitative determination as to whether the cells in the tissue were still viable, and this was determined visually by a purple colour change but was not quantified.

Thereafter, supernatants were harvested into 2 mL cryovials and stored at -80°C. Cervical explants were harvested in 800 μL QIAzol® in 2 mL cryovial tubes (Nunc, Germany) and were subsequently homogenised using a hand-held homogeniser (TissueRuptor®, Qiagen, Netherlands) with disposable probes (TissueRuptor Probes, Qiagen, Netherlands). Samples were homogenised for 20 second pulses, for up to 1 minute on ice. Homogenates were transferred to new microfuge tubes and incubated for 5 minutes at room temperature. RNA was isolated using the RNeasy® Microarray Tissue Mini Kit (Qiagen, Netherlands), according to the manufacturer’s instructions. RNA was assessed for quality and quantity, and run on a denaturing agarose gel as previously described in section 2.7.1.

2.13 Statistical Analysis

Results were analysed using GraphPad PRISM (version 7) software from GraphPad Software Inc (La Jolla California, USA). Where samples were treated with ligands at one time point, a one-way ANOVA, with a Tukey multiple comparison post-test (comparing each group to every other group), was performed. For experiments that had two different treatments, a two-way ANOVA was performed with a Tukey multiple comparison post-test (comparing all groups to each other or to a control). Data were plotted as mean ± SEM on histograms, with n values represented in each figure legend. For dose response curves, data was analysed with vehicle control set to 100%, then a stimulatory or inhibitory dose response curve with a non-linear regression model was employed using “log agonist vs response (variable slope) curve with the Hill slope set to 1. Where statistical significance is obtained, significance is denoted by *, **, ***, or **** to indicate p<0.05, p<0.01, p<0.001, or p<0.0001 respectively. Statistical significance is also represented using letters where bars with the same letter are not significantly different to each other, while bars with different letters denote statistical significance.
Chapter 3: Results

3.1 Progestins differentially regulate expression of endogenous pro- and anti-inflammatory genes in TZM-bl cervical cells

As a result of the concerns about MPA increasing the risk of HIV infection, other progestins may be more suitable for use in areas of high HIV risk. Previous studies have shown that MPA, unlike NET, regulates the expression of immune function genes via the GR (Govender et al., 2014); however, very little information is available for other progestins including LNG, NES and ETG. In the present study, the effect of a panel of progestins on the expression of genes known to be regulated by the GR was investigated in TZM-bl cervical cells, which express endogenous GR protein. It has been well established in previous studies that the inflammatory genes, IL-8, IL-6 and GILZ are regulated by the GR (Govender et al., 2014, Hapgood et al., 2014b, Koubovec et al., 2004, Ronacher et al., 2009) and thus these were selected for investigation in the current study. GILZ and IL-6 are widely known to be classic examples of a GR transactivation model (GLZ) and a transrepression model (IL-6). To determine the effect of the progestins, TZM-bl cells were stimulated with the different progestins (MPA, NET, LNG, NES, ETG) and the GR agonist dexamethasone (DEX) at a concentration of 100 nM for 24 hrs, with EtOH as the vehicle control. The glucocorticoid DEX was included in the panel as a positive control to compare if any of the other progestins would act via the GR. Progestins were used at 100 nM and this was done in order to compare them to MPA, which has been shown to repress many cytokines and chemokines at concentrations ranging from 1 – 100 nM via the GR (Govender et al., 2014, Koubovec et al., 2005, Ronacher et al., 2009).

Relative mRNA expression of the pro-inflammatory IL-6 and IL-8 genes, along with the anti-inflammatory GILZ and the house keeping gene, GAPDH, was measured by qRT-PCR. Importantly, RNA purity and quality was assessed by spectrophotometry and RNA integrity was assessed on a denaturing formaldehyde agarose gel. Only RNA with an absorbance 260/280 ratio >1.8 and a 260/230 ratio between 2 and 2.2 was used. Intact 28S,18S and the smaller 5S ribosomal bands were clearly visible on the gel, indicating that good quality RNA was isolated effectively and was appropriate to use for qRT-PCR (Figure 3.1 a). Treatment with the progestins MPA, LNG, NES and ETG significantly repressed IL-6 mRNA levels, when compared to vehicle, while the apparent IL-6 repression with NET was not significant (Figure 3.1 b). DEX resulted in the greatest percentage repression of IL-6 expression, which is consistent with previous findings of Govender et al. in HeLa and End1E6/E7 cells (Govender et al., 2014). Unlike for IL-6 expression, all progestins, as well as DEX appeared to slightly upregulate IL-8 mRNA, although this effect was not significant (Figure 3.1 c), suggesting that these ligands may be inducing a pro-inflammatory IL-8 response.
In contrast to the pro-inflammatory genes, there was a significant 10-fold induction of the known GR transactivation GILZ gene, following treatment with DEX, as expected. MPA and NES treatment induced GILZ mRNA 5-fold, although this effect was not statistically significant. The other progestins, NET, LNG, and ETG also appeared to induce GILZ mRNA levels, but to a lesser extent than DEX, MPA or NES and the effect was not statistically significant (Figure 3.1 d). These results show that progestins have immunosuppressive effects on the immune function genes IL-6 and GILZ, and appear to elicit a pro-inflammatory IL-8 response, although this was not statistically significant. MPA acted similarly to the GR agonist DEX on IL-6 and GILZ gene expression, albeit showing a lower response than DEX. This was consistent with MPA being a partial GR agonist and with previous reports showing that MPA regulates inflammatory gene expression in endocervical cells via the GR (Govender et al., 2014). NES showed similar responses to MPA by significantly repressing IL-6 and appearing to partially induce GILZ. This result suggests that NES may have partial glucocorticoid-like properties, as MPA does.

Figure 3.1 Progestins differentially regulate IL-6 and GILZ mRNA expression, while having no significant effect on IL-8. TZM-bl cells were seeded into 12-well plates at a density of 1 x 10⁵ cells/well, and incubated for 24 hrs. Thereafter, cells were treated with 100 nM DEX, MPA, NET, LNG, NES and ETG. EtOH (0.1% v/v) was used as a vehicle control, and the cells were then incubated for 24 hrs. Total RNA was isolated and reverse-transcribed to make cDNA. A) Assessment of RNA quality on a
denaturing formaldehyde agarose gel. A representative image of one experiment is shown. Relative expression of (B) IL-6, (C) IL-8 and (D) GILZ mRNA was measured by qRT-PCR and normalised to GAPDH mRNA. Relative fold mRNA expression was normalized to vehicle set to 1. Graphs show pooled results of at least three independent experiments. Statistical analysis was done using a one-way ANOVA, followed by Tukey’s multiple comparison post-test. Statistical significance is denoted by ** or **** to indicate P< 0.01 or P< 0.0001, respectively when comparing the control to treated conditions.

3.2 The ARVs TDF and DPV differentially regulate expression of select endogenous pro-inflammatory genes in cervical cells at physiologically relevant concentrations

3.2.1 High concentrations of DPV, unlike TDF, reduce cell viability of cervical cells

The ARVs, TDF and DPV are currently being investigated for combination strategies with hormonal contraception. Although TDF and DPV have in vitro IC50 concentrations in the nanomolar range against wild type HIV-1, they are currently being investigated for intravaginal delivery at micromolar concentrations (Keller et al., 2016, Romano et al., 2009). However, whether they are able to regulate expression of endogenous inflammatory genes at any concentration is not well known. To determine whether TDF and DPV have any effect on endogenous inflammatory gene expression after 24 hrs, dose response experiments were performed with increasing doses of DPV (10^{-10} to 10^{-6} M) or TDF (10^{-7} to 10^{-5} M) and compared to DMSO as the vehicle control. Cell viability was assessed in parallel using an MTT assay to determine if the concentrations used were toxic to the cells. 10 µM (10^{-5} M) DPV significantly reduced cell viability by over 60%, compared to vehicle, while 1 µM (10^{-6} M) DPV reduced cell viability by approximately 20%, but this was not significant (Figure 3.2 a). Doses lower than 1 µM DPV did not significantly reduce cell viability. TDF had no effect on cell viability at all concentrations used (Figure 3.2 b).

![Graphs showing cell viability](image)

**Figure 3.2:** DPV, unlike TDF, reduces viability of TZM-bl cells at high concentrations. TZM-bl cells were seeded into 96-well plates at a density of 1 x 10^4 cells/well and incubated for 24 hrs. Thereafter, cells were treated with different concentrations of (A) DPV and (B) TDF, ranging from 10^{-10} – 10^{-5} M, in
triplicate. DMSO (0.1% v/v) was used as the vehicle control, and the cells were then incubated for 24 hrs. Cells were then treated with MTT reagent, and after solubilisation, the absorbance was read at 595 nm. Cell viability was normalized to the vehicle control, which was set to 100%. Graphs show pooled results of three independent experiments, with each condition done in triplicate. Statistical analysis was done using a one-way ANOVA, followed by Tukey’s multiple comparison post-test. Statistical significance is denoted by * to indicate P< 0.01 when comparing the control to treated conditions.

3.2.2 DPV, unlike TDF, upregulates the pro-inflammatory IL-6 and IL-8 genes.

Gene expression analysis showed that DPV resulted in a 4-fold induction of IL-6 mRNA expression (Figure 3.3 a) and a 7-fold induction of IL-8 mRNA expression at a concentration of 1 µM (Figure 3.3.c), while having no effect at lower concentrations. In comparison to DPV, TDF had no significant effect on IL-6 mRNA expression; however, it did appear to increase IL-6 mRNA expression at a concentration of 100 nM and 1 µM (Figure 3.3 b). TDF had no effect on IL-8 mRNA expression at all concentrations after 24 hrs (Figure 3.3 d). DPV and TDF had no significant effects on anti-inflammatory GILZ expression after 24 hrs (Figure 3.3 e and f). These results show that high concentrations of DPV, unlike TDF, exert both cytotoxic effects on cervical cells and upregulate IL-6 and IL-8 pro-inflammatory genes in TZM-bl cervical cells, while having no effect on mRNA levels of the anti-inflammatory GILZ gene.
Figure 3.3: DPV, unlike TDF, upregulates pro-inflammatory mRNA levels in TZM-bl cervical cells, while having no effect on anti-inflammatory GILZ mRNA. TZM-bl cells were seeded into 12-well plates at a density of $1 \times 10^5$ cells/well, and incubated for 24 hrs. Thereafter, cells were treated with different concentrations of DPV ranging from $10^{-10}$ to $10^{-6}$ M, and different concentrations of TDF ranging from 100 nM to 10 µM, for 24 hours. DMSO (0.1% v/v) was used as the vehicle control. Total RNA was isolated and reverse-transcribed to make cDNA. Relative expression of (A, B) IL-6, (C, D) IL-6 and (E, F) GILZ mRNA was measured by qRT-PCR, and normalised to GAPDH mRNA. Relative fold mRNA expression was normalized to the vehicle control set to 1. Graphs show pooled results of three independent experiments. Statistical analysis was done using a one-way ANOVA, followed by Tukey's multiple comparison post-test. Statistical significance is denoted by *** to indicate $P < 0.001$ when comparing the control to treated conditions.

3.3 ARV-progestin combinations alter select endogenous pro-inflammatory immune function gene expression

3.3.1 MPA potentiates the pro-inflammatory effects of DPV on IL-8 mRNA expression

An important consideration for multipurpose strategies is to determine the combined effect of progestins and ARVs on immune function gene regulation as this may contribute to an altered immune state in the female genital tract. As shown in figure 3.1, MPA exerted the greatest gene specific effects on inflammatory genes, compared to the other progestins, and acted similarly to the synthetic GR agonist, DEX in a transactivation model gene (GILZ) and a transrepression model gene (IL-6). Hence, the effect of an ARV on MPA-induced responses of GR regulated genes, was investigated, and DEX was used as the positive control. TZM-bl cells were treated with 100 nM DEX and MPA and 1 µM DPV and TDF alone, and then co-stimulated with the combinations (DEX and TDF, DEX and DPV; MPA and TDF, MPA and DPV) for 24 hrs. Subsequently, mRNA levels of the immunoregulatory genes were measured using qRT-PCR.

The previous result in figure 3.1 c showed that all the progestins, including DEX, induced a slight upregulation of IL-8 mRNA levels. In the combination experiments, incubations with DEX
and MPA alone, also appeared to upregulate IL-8 mRNA levels (Figure 3.4 a), although, as in figure 3.1 this effect was not significant. 1 µM DPV significantly upregulated IL-8 mRNA by 11-fold, as previously observed in figure 3.3 c, and upon co-stimulation with MPA, this response was potentiated (16-fold), which was not seen following co-stimulation with DEX. 1 µM TDF had no effect on IL-8 expression as previously observed in figure 3.3 d. In comparison to DEX and MPA, co-stimulation with TDF did not significantly alter the IL-8 response.

3.3.2 MPA represses the DPV mediated upregulation of IL-6 mRNA expression

Gene expression analysis of IL-6 showed that DEX and MPA alone appeared to repress IL-6 mRNA expression, although this repression was not significant, while 1 µM DPV alone significantly upregulated IL-6 mRNA (Figure 3.4 b). Co-stimulation of DEX or MPA with DPV resulted in a significant repression of the large DPV-induced 11-fold increase of IL-6 mRNA expression to near vehicle levels (Figure 3.4 b). TDF alone had no effect on IL-6 expression as was previously seen in figure 3.3 b. In comparison to DEX and MPA alone, the presence of TDF resulted in no significant changes to DEX or MPA effects on IL-6 mRNA levels (Figure 3.4 b). The presence of DPV in combination with DEX or MPA appeared to lift the strong repression seen with DEX and MPA, but this effect also failed to reach significance, possibly due to the large number of variables and the statistical power of the experiment being too small to detect significant differences. This compared to figure 3.1, where fewer variables were used and statistically significant differences were obtained for IL-6 repression.

3.3.3 DPV and TDF in combination with MPA or DEX have no effect on mRNA levels of the anti-inflammatory GILZ immune function gene

Similar to the result in figure 3.1 d, MPA upregulated GILZ mRNA (Figure 3.4 c) and acted similarly to the GR agonist, DEX as previously seen. Although the increase was not significant, it closely approached statistical significance (p-value 0.077). The presence of TDF and DPV had no effect on the ability of DEX and MPA to upregulate GILZ expression (Figure 3.4 c).

3.3.4 DPV enhances IL-6 secretory protein levels in the absence and presence of DEX and MPA

To determine whether the effects (seen in figure 3.4) of DEX, MPA, TDF and DPV alone and in combination, on pro-inflammatory mRNA levels are reflected at the protein level, the levels of secreted IL-8 and IL-6 protein were measured. Supernatants were collected from TZM-bl cells following 24 hr treatment and cytokine levels were measured using the R&D Systems DuoSet® ELISA kit. Similar to the mRNA expression results in figure 3.4 b, both DEX and
MPA resulted in an apparent repression of IL-6 secreted protein (Figure 3.5) but did not reach significance.

Figure 3.4: The ARVs, DPV and TDF, have differential effects on select immune function genes in the absence and presence of DEX and MPA. TZM-bl cells were seeded into 12-well plates at a density of \(1 \times 10^5\) cells/well, and incubated for 24 hrs. Thereafter, cells were treated with 100 nM DEX and MPA, and 1 µM TDF and DPV. EtOH (0.1% v/v) was used as the vehicle control for the progestins (vehicle 1), and DMSO (0.1% v/v) was used as the vehicle control for the ARVs (vehicle 2). Cells were also treated with combinations of these (DEX and TDF, DEX and DPV; MPA and TDF, MPA and DPV)
and ETOH+DMSO (0.1% v/v for each) was used as the vehicle control (vehicle 3). Total RNA was harvested, isolated and reverse-transcribed to make cDNA. Relative expression of (A) IL-8, (B) IL-6 and (C) GILZ mRNA was measured by qRT-PCR and normalized to GAPDH mRNA expression. Relative fold mRNA expression was normalized to basal activity, with the ETOH+DMSO vehicle set to 1. Graphs show pooled results of at least three independent experiments. Statistical analysis was done using a two-way ANOVA, followed by Tukey’s multiple comparison post-test. Statistical significance is denoted by *, **, ***, or **** to indicate P < 0.05, P < 0.01, P < 0.001 or P < 0.0001, respectively when comparing each treatment to all other treated conditions. Statistical significance is also represented using letters where bars with the same letter are not significantly different to each other, while bars with different letters denote statistical significance.

TDF did not alter the secreted levels of IL-6 protein, while treatment with DPV significantly increased secreted IL-6 protein levels by 2-fold. Co-stimulation of DEX or MPA with DPV significantly reduced the DPV-induced increase of secreted IL-6 protein levels, reflecting a similar response to that on IL-6 mRNA expression (Figure 3.4 b). As previously observed for mRNA levels in figure 3.4 b, DPV significantly lifted the MPA-induced repression of IL-6 protein. Repression of IL-6 protein levels by DEX and MPA appeared to persist in the presence of TDF, further indicating that TDF does not appear to alter the expression of pro-inflammatory genes. The detection of secreted IL-8 protein levels by ELISA was not reproducible, possibly due to low levels of IL-8 protein secreted by TZM-bl cells or insufficient sensitivity of the assay and thus, a clear result was not obtained for IL-8 protein levels.

Figure 3.5: DPV upregulates IL-6 secreted protein levels and MPA and DEX reduce this response. TZM-bl cells were seeded into 12-well plates at a density of 1 x 10^5 cells/well and incubated for 24 hrs. Thereafter, the cells were treated with 100 nM DEX and MPA, and 1 µM TDF and DPV. Cells were also treated with combinations of these (DEX and TDF, DEX and DPV; MPA and TDF, MPA and DPV) with ETOH+DMSO (0.1% v/v for each) as the vehicle control and were then incubated for 24 hrs. Supernatants were then collected prior to cell harvest and cytokine protein levels for IL-6 were determined by using the R&D Systems DuoSet® ELISA kit. Relative protein expression was normalized.
to the vehicle control set to 1 to obtain relative fold change. Graphs show pooled results of three independent experiments, with each condition done in duplicate. Statistical analysis was done using a two-way ANOVA, followed by a Tukey multiple comparison post-test, comparing each treatment to all other treated conditions. Statistical significance is denoted by *, ** or *** to indicate P< 0.05, P< 0.01 or P< 0.001, respectively. Statistical significance is also represented using letters where bars with the same letter are not significantly different to each other, while bars with different letters denote statistical significance.

Taken together these results indicate that DPV, unlike TDF, increases pro-inflammatory IL-8 and IL-6 mRNA expression, as well as IL-6 protein levels. The presence of MPA potentiates the DPV-induced, IL-8 mRNA response, while the presence of DEX or MPA significantly reduces the IL-6 response at both mRNA and protein levels. At the protein level, DPV reduced the immunosuppressive ability of MPA.

3.4 Progestins alone or in combination with ARVs appear to affect endogenous AR and GR steroid receptor protein levels

3.4.1 Select progestins appear to alter AR and GR protein levels after 24 hrs

Having shown that the progestins and ARVs alter expression levels of immune function genes regulated by steroid receptors, the effect of progestins and ARVs on endogenous steroid receptor protein levels was investigated in TZM-bl cervical cells by Western blot analysis. GR, AR and PR receptor levels were investigated and representative blots are shown. GAPDH was used as a loading control and relative receptor levels were quantified and normalized to the vehicle. COS-1 cell lysates from cells transfected to overexpress each receptor were used as positive controls for each protein. COS-1 cell lysates after transfection with empty vector (pCDNA) were used as a negative control. The results showed that TZM-bl cells express detectable AR and GR protein, but not PR protein (Figure 3.6).

Previous reports have shown that MPA, like DEX, affects GR activity and GR protein levels (Avenant et al., 2010b). MPA has also been shown to affect AR activity and AR protein levels (Apparao et al., 2002). Few studies; however, have investigated the effects of other progestins like NET, LNG, NES and ETG on GR and AR levels. In this study, treatment with the progestins showed varied effects on GR and AR receptor levels. Upon quantification of GR levels, DEX, MPA, NES and ETG appeared to reduce GR protein levels, while NET and LNG did not have any apparent effect on GR protein (Figure 3.6 a). These reductions were not significant. In comparison, LNG appeared to increase AR protein levels and this effect was approaching significance with a p-value of 0.071 (Figure 3.6 b). NET and ETG similarly appeared to increase AR receptor levels, while DEX and NES appeared to reduce AR levels, although following quantification this observation was not significant.
Figure 3.6: LNG and NET appear to increase AR protein while DEX, MPA and NES appear to reduce both AR and GR protein levels. TZM-bl cells were seeded into 12 well plates at a density of 1 x 10^5 cells/well and incubated for 24 hrs. Thereafter cells were treated with 100 nM DEX, MPA, NET, LNG, NES and ETG. EtOH (0.1% v/v) was used as a vehicle control and the cells were then incubated for 24 hrs. For positive controls, COS-1 cells were transfected with steroid receptor expression vectors (pSV-hAR, and pcDNA3 GR WT) and the empty expression vector pcDNA3 was used as the negative control. Total protein was harvested, and equal amounts of whole cell lysates were loaded on SDS page gels and analyzed by Western blotting with antibodies specific for (A) GR (95 kDa) and PR (114 kDa) as well as (B) AR (110 kDa). GAPDH (37 kDa) was used as the loading control. Single representative blots are shown and graphs show quantified pooled results of at least three experiments. Relative AR and GR levels were normalized to the vehicle control set to 1 to obtain relative fold change. Statistical analysis was done using a one-way ANOVA, followed by Tukey’s multiple comparison post-test.
3.4.2 TDF and DPV appear to alter GR and AR protein levels in the absence and presence of MPA and DEX.

Whether ARVs regulate steroid receptor levels is not yet known and having shown that DPV alters the MPA-induced IL-6 and IL-8 responses (Figure 3.4), the effect of TDF and DPV in the absence and presence of DEX and MPA on the levels of the GR and AR was next determined. The effect of DPV alone and in combination with DEX and MPA on AR and GR protein levels is depicted in Figure 3.7. DPV appeared to reduce total protein content as both the GR and GAPDH bands were reduced (Figure 3.7 a). However, the ratio of GR to GAPDH was not significantly affected. The combinations of DEX or MPA with DPV had no large overall effect on GR levels (Figure 3.7 a); however, in the presence of DEX and DPV, GR levels appeared to be reduced more dramatically, when compared to DEX alone, and GAPDH levels were not affected by the combination. After quantification the reduction observed with DEX and DPV was not significant. A band of similar size to the GR was present in the AR positive control lane in figure 3.7 a, and this was most likely due to low levels of endogenous GR found in COS-1 cells, which were used to make the positive controls.

Changes in AR levels are depicted in Figure 3.7 b. Similar to what was observed with GR levels, DPV once again appeared to reduce total protein content as both the AR and GAPDH protein bands were reduced (Figure 3.7 b). DEX and MPA in combination with DPV had no overall effect on AR protein levels, although a significant decrease was observed with DEX in combination with DPV when compared to the vehicle 2 control (DMSO). Results for GR and AR were similar and showed that DPV may reduce total protein content and that DPV in combination with DEX or MPA may reduce GR or AR protein.

GR levels also appeared to be reduced in the presence of TDF and MPA as indicated on the representative blot, but pooling quantified blots of all experiments, did not show statistical significance of this effect (Figure 3.8 a). TDF also had no significant effect on AR levels. As shown previously in figure 3.6 a, DEX and MPA showed no significant effect on AR levels (Figure 3.8 b). Similarly, TDF in combination with DEX or MPA had no overall effect on AR levels. Thus, these results show GR turnover by DEX alone and in the presence of TDF, but show no significant effects of TDF alone and in combination with MPA on AR and GR receptor levels.
Figure 3.7: DPV alone and in combination with DEX appears to alter AR and GR levels. TZM-bl cells were seeded into 12-well plates at a density of $1 \times 10^5$ cells/well and incubated for 24 hrs. Thereafter, cells were treated with 100 nM DEX and MPA, and 1 µM DPV. EtOH (0.1% v/v) was used as the vehicle control for the progestins (vehicle 1), and DMSO (0.1% v/v) was used as the vehicle control for the ARVs (vehicle 2). Cells were also treated with combinations of these (DEX and DPV; MPA and DPV) and EtOH+DMSO (0.1% v/v for each) was used as the vehicle control (vehicle 3). For positive controls, COS-1 cells were transfected with 1 µg/well steroid receptor expression vectors (pSV-hAR, and pcDNA3 GR WT) and the empty expression vector pCDNA was used as the negative control. Total protein was harvested, and equal amounts of whole cell lysates were loaded on SDS page gels and analyzed by Western blotting with antibodies specific for (A) GR (95 kDa) and (B) AR (110 kDa) and GAPDH (37 kDa), which was used as the loading control. Single representative blots are shown and graphs show quantified pooled results of at least three experiments. Relative AR and GR levels were normalized to the EtOH+DMSO vehicle control set to 1 to obtain relative fold change. Statistical analysis was done using a one-way ANOVA, followed by Tukey’s multiple comparison post-test, comparing each treatment to all other treated conditions. Statistical significance is denoted by * to indicate P< 0.05.
Taken together, these results show that the ARVs, TDF or DPV in combination with the glucocorticoid, DEX, have significant effects on endogenous AR and GR receptor protein levels. The reduction of total protein content by 1 µM DPV may be due to the small decrease seen in cell viability (Figure 3.2 a) and thus, related to its cytotoxic effects. There were large error bars following quantification and pooling of all experiments and this may have been due to the large variation between Western blot repeats. This led to the statistical power of the experiments not being sufficient to obtain statistically significant differences. The large number of variables in each experiment and technical error could have contributed to the low statistical power of the experiments. Several steps in the analysis could have introduced technical error such as transfer and detection of antibodies on the membrane as well as differences in exposure times of the membrane to film. General trends were observed; however, further repeats using fewer variables are needed to determine whether differences are statistically significant.

3.5 DPV exerts effects on the transactivational activity of over-expressed AR but not GR

Results from this study have shown that DPV upregulates IL-8 and IL-6 expression in TZM-bl cells (Figure 3.3). Although the ARVs do not appear to greatly alter steroid receptor levels, their ability to alter steroid receptor activity has not been directly investigated. To determine this, the effect of TDF and DPV on GR and AR transactivational activity was investigated. Expression plasmids for the GR (pcDNA3 GR WT), the AR (pSV-AR) and the control plasmid with no steroid receptor (pcDNA3) were first prepared and purified to obtain pure and clean plasmid DNA. Plasmid identity and integrity was confirmed by restriction enzyme digestion (Appendix Figure 1).

3.5.1 TDF and DPV have no significant effect on GR transactivational activity

The effect of TDF and DPV on the activity of exogenously expressed GR in the absence and presence of the GR agonist, DEX, was investigated. U2OS cells were co-transfected with the pcDNA3 GR WT plasmid or the control pcDNA3 plasmid, and the reporter plasmid pTAT-GRE.
Figure 3.8: TDF in combination with DEX appears to alter GR, but not AR levels. TZM-bl cells were seeded into 12-well plates at a density of 1 x 10^5 cells/well and incubated for 24 hrs. Thereafter, cells were treated with 100 nM DEX and MPA, and 1 μM TDF. EtOH (0.1% v/v) was used as the vehicle control for the progestins (vehicle 1), and DMSO (0.1% v/v) was used as the vehicle control for the ARVs (vehicle 2). Cells were also treated with combinations of these (DEX and TDF; MPA and TDF) and ETOH+DMSO (0.1% v/v for each) was used as the vehicle control (vehicle 3). For positive controls, COS-1 cells were transfected with 1 μg/well steroid receptor expression vectors (pSV-hAR, and pcDNA3 GR WT) and the empty expression vector pCDNA was used as the negative control. Total protein was harvested and equal amounts of whole cell lysates were loaded on SDS page gels and analyzed by Western blotting with antibodies specific for (A) GR (95 kDa) and (B) AR (110 kDa). GAPDH (37 kDa) was used as the loading control. Single representative blots are shown and graphs show quantified pooled results of at least three experiments. Relative AR and GR levels were normalized to the ETOH+DMSO vehicle control set to 1 to obtain relative fold change. Statistical analysis was done using a two-way ANOVA, followed by Tukey’s multiple comparison post-test, comparing each treatment to all other treated conditions. Statistical significance is denoted by * or ** to indicate P< 0.05 or P< 0.01, respectively.
Cells were subsequently stimulated with 1 µM TDF or DPV in the absence and presence of 100 nM DEX. Over-expression of the GR was confirmed by Western blot as shown in figure 3.9 a. Low levels of endogenous GR were detected in cell lysates transfected with the empty vector (pcDNA).

Figure 3.9: Effect of TDF and DPV on transactivational activity of exogenously expressed GR. U2OS cells were seeded into 10 cm plates at a density of 1.5 x 10^5 and incubated for 24 hrs. Thereafter, cells were transiently transfected for 24 hrs with pTAT-GRE, pcDNA-3 (empty vector) or pCDNA3 GR WT. Cells were then replated into 96 well plates at a density of 1 x 10^4 and incubated for 24 hrs. Then, the cells were treated with 1 µM TDF or DPV in the absence or presence of 100 nM DEX. EtOH + DMSO (0.1% v/v for each; vehicle) was the vehicle control and cells were incubated for 24 hrs. A) Equal volumes of lysate for pcDNA3 transfected and pCDNA3 GR WT transfected cells were analysed by Western blotting with antibodies against the GR and GAPDH as the loading control. B) Luciferase activity was normalised to protein content per well as determined by Bradford assay. Luciferase activity for TDF and DPV was normalized to the vehicle control (EtOH+DMSO) to obtain a relative fold induction. Graphs show pooled results of at least three independent experiments with each condition done in triplicate. Statistical analysis was done using a two-way ANOVA, followed by a Tukey multiple comparison post-test, comparing each treatment to all other treated conditions. Statistical significance is represented using letters where bars with the same letter are not significantly different to each other, while bars with different letters denote statistical significance.

TDF and DPV appeared to repress GR activity in the absence of DEX although this was not significant, while there was no effect observed in the presence of DEX for both ARVs (Figure 3.9 b). Further repeats using fewer variables are needed to confirm the apparent ARV repression of GR activity in the absence of DEX, as this would increase the statistical power of the experiment. Overall, TDF and DPV did not have any significant effect on GR transactivation.
3.5.2 DPV, unlike TDF, has ligand-dependent effects on AR transactivational activity

The ability of ARVs to activate exogenously expressed AR, in the absence and presence of an AR agonist, mibolerone (MIB), was then investigated. U2OS cells were co-transfected with the pSV-AR plasmid or the control pcDNA3 plasmid, along with the Luciferase reporter plasmid pTAT-GRE. Cells were subsequently stimulated with two concentrations of TDF or DPV, 100 nM and 1 µM, in the absence and presence of 0.01 nM of the AR agonist, MIB. Confirmation of AR expression in U2OS cells is depicted in Figure 3.10 a. In comparison to the control pcDNA3 transfected cells, AR expressing cells showed significant AR transactivation in the presence of MIB (Figure 3.10 b). In AR expressing cells, in the absence of MIB, 1 µM TDF showed an apparent increase compared to the vehicle (Figure 3.10 b), indicating that TDF may have ligand independent effects on AR activity at higher concentrations, although this effect did not reach significance. In the AR expressing cells, MIB significantly increased AR activity, but this activity was not significantly affected by any TDF concentrations, which all showed very large error. Similarly to TDF, DPV in AR expressing cells, in the absence of MIB, appeared to slightly increase AR activity (Figure 3.10 c), although this effect was not significant suggesting that DPV may also have small ligand independent effects. Interestingly, when 100 nM DPV was added to AR expressing cells, in the presence of MIB, it showed a significant increase in AR activity, when compared to the vehicle. The same effect was not seen with 1 µM DPV. This result suggests that at a low concentration of MIB (0.01 nM), different concentrations of DPV have differential effects on the efficacy of MIB on AR activity.

This data shows that both TDF and DPV may have some ligand independent effects on AR activity, while only DPV has statistically significant effects on AR activity in the presence of the AR ligand, MIB. Use of fewer variables and more biological repeats are required to obtain statistical differences of the ligand independent effects of TDF and DPV on the AR.
Figure 3.10: Effect of TDF and DPV on transactivational activity of AR. U2OS cells were seeded into 10 cm plates at a density of $1.5 \times 10^5$ and incubated for 24 hrs. Thereafter, cells were transiently transfected for 24 hrs with pTAT-GRE, pcDNA-3 (empty vector) or pSV-hAR. Cells were then replated into 96 well plates at a density of $1 \times 10^4$ and in 12 well plates at a density of $1.5 \times 10^5$ for Western blot analysis. Subsequently cells were treated with 100 nM and 1 µM TDF or DPV in the absence or presence of 0.01 nM MIB. EtOH+DMSO (0.1% v/v for each) was used as the vehicle control and cells were incubated for 24 hrs. A) Equal volumes of lysate for pSV-AR transfected and pcDNA3 transfected cells were analysed by Western blotting with antibodies against the AR and GAPDH as the loading control. Cells were harvested with 1 X Reporter lysis buffer and the luciferase activity was measured. Luciferase activity was normalized to protein content per well as determined by Bradford assay. Luciferase activity for B) TDF and C) DPV was normalized to the vehicle control (EtOH+DMSO) to obtain a relative fold induction. Graphs show pooled results of at least three independent experiments with each condition done in triplicate. Statistical analysis was done using a two-way ANOVA, followed by a Tukey multiple comparison post-test. Statistical significance is denoted by *, ** or **** to indicate $P< 0.05$, $P<0.001$ or $P< 0.0001$, respectively when comparing each treatment to all other treated conditions. Statistical significance is also represented using letters where bars with the same letter are not significantly different to each other, while bars with different letters denote statistical significance.

3.5.3 DPV, unlike TDF, significantly alters the efficacy and potency of mibolerone

Biological responses of steroid receptor regulated genes depend largely on the efficacy and potency of the steroid receptor ligands. To our knowledge no studies have investigated the effect of ARVs on the potency and efficacy of steroid receptor ligands. Having shown in figure 3.10 that 100 nM DPV but not TDF has a significant effect on AR transactivational activity in the presence of one concentration of the AR agonist, MIB, the effect of ARVs on the efficacy and potency of MIB was further investigated at varying doses of MIB. U20S cells were transfected as previously described and then stimulated with increasing doses of MIB ranging from $10^{-12}$ – $10^{-8}$ M in the absence and presence of 1 µM TDF or DPV. Confirmation of AR expression in U20S cells is depicted in Figure 3.11 a. Unlike in figure 3.10, one concentration
of ARV (1 µM) was used in these experiments and this was because the higher concentration represents the high intravaginal concentrations in women. The dose response curves of cells transfected with AR are depicted in Figure 3.11. No effect on the efficacy and potency of MIB in U20S cells was seen in the presence of TDF (Figure 3.11 b). In contrast, DPV significantly decreased the efficacy and significantly increased the potency of MIB by left-shifting the dose response curve and reducing the EC$_{50}$ from 7.8 nM to 0.018 nM with a p-value of 0.0109. (Figure 3.11 c).

In figure 3.10 c, 1 µM DPV did not significantly increase AR activity in the presence of 0.01 nM MIB and this finding was consistent in the MIB + DPV dose response curve. In the presence of 1 µM DPV, the maximal MIB response was reached at a lower concentration (0.1 nM), compared to in the absence of DPV (100 nM), suggesting that higher concentrations of DPV may make AR ligands at concentrations ranging from 0.1 – 10 nM more potent (Figure 3.11 c). In figure 3.10 c, a lower concentration of DPV (100 nM) was sufficient to increase the MIB response at 0.01 nM; suggesting that lower concentrations of DPV may make cells more sensitive to AR activity, when very low concentrations of AR ligands are present in the cells. This data shows that DPV unlike TDF has a dramatic effect on the efficacy and potency of AR ligands, and may contribute to different off target side effects via the AR steriod receptor activity.

a)
Figure 3.11: Effect of TDF and DPV on efficacy and potency of mibolerone. U2OS cells were seeded into 10 cm plates at a density of $1.5 \times 10^5$ and incubated for 24 hrs. Thereafter, cells were transiently transfected for 24 hrs with pTAT-GRE, pcDNA-3 (empty vector) or pSV-hAR. Cells were then replated into 96 well plates at a density of $1 \times 10^4$ and in 12 well plates at a density of $1.5 \times 10^5$ for Western blot analysis for 24 hrs. Then, the cells were treated with increasing concentrations of MIB (0.001 nM – 10 nM) in the absence or presence of 1 µM TDF and DPV. EtOH+DMSO (0.1% v/v for each) was used as the vehicle control and cells were incubated for 24 hrs. A) Equal volumes of lysate for pSV-AR transfected and pcDNA3 transfected cells were analysed by Western blotting with antibodies against the AR and GAPDH as the loading control. Cells were harvested and the luciferase activity was measured. Luciferase activity was normalized to protein content per well as determined by Bradford assay. Luciferase activity for B) TDF and C) DPV was normalized to the vehicle control (EtOH+DMSO) in order to obtain a relative fold induction. Statistical analysis was done using a non-linear regression model with a log agonist vs. response (variable slope) curve, with the slope set to 1. Comparison of the best fit top, bottom and EC50 values of the curves for each data set, was performed, to obtain statistical significance differences between the curves.
3.6 ARVs and progestins alone and in combination differentially regulate HIV replication in cervical cells

3.6.1 DEX and MPA appear to increase viral replication at higher viral amounts

The concurrent use of hormonal contraceptives such as MPA with ARVs, may raise concerns of MPA possibly interfering with the anti-HIV activity of ARVs, most especially since MPA is associated with an increased risk of HIV acquisition. In light of this, the effect of the progestins alone and in combination with TDF and DPV on HIV-1 replication, was investigated. HIV-1\textsubscript{Bal\_Renilla} infectious molecular clones coding for R5 tropic virus, were prepared in HEK293T cells. To determine the TCID50, the virus was titrated in TZM-bl cells, which are engineered to express the HIV receptor CD4, as well as CCR5 and CXCR4 HIV co-receptors. TZM-bl cells also contain the HIV long terminal repeat (LTR) in their genome and are thus a good indicator cell for HIV replication. The TCID50 of the virus prepared was calculated to be 69183.1 IU/mL.

After pre-treatment with DEX and MPA for 2 hrs, TZM-bl cells were infected with different concentrations of virus ranging from 5 – 200 IU/well. DEX and MPA appeared to increase viral replication from virus concentrations of 40 IU up to 200 IU (Figure 3.13), showing that DEX and MPA increase viral replication in a virus dose-dependent manner in TZM-bl cervical cells.

![Figure 3.13: DEX and MPA appear to increase viral replication at higher viral amounts.](image)

TZM-bl cells were seeded into 96-well plates at a density of 1 x 10\(^4\) cells/well and incubated for 24 hrs. Thereafter cells were treated with 100 nM DEX and MPA with EtOH (0.1% v/v) as a vehicle control for 2 hours. Cells were then infected with the following amounts of infectious units of HIV-1\textsubscript{Bal\_Renilla} 5, 10, 20, 40, 100, and 200 or virus control per well and were incubated for 48 hrs. Thereafter, the cells were lysed and the luciferase activity was measured. In addition, a parallel experiment was done and MTT activity was measured. Luciferase activity was normalized to MTT activity. Furthermore, relative LTR activity/MTT was normalized to + HIV-1 basal activity (EtOH) for each virus amount set to 1. Graphs show pooled results of two independent experiments with each condition done in triplicate.
3.6.2 DEX and MPA in combination with TDF or DPV do not alter antiviral activity of ARVs

The ARVs function to inhibit virus replication and whether progestins are able to interfere with that ability is an important question for women, as the number of people using ARVs and hormonal contraceptives together is likely to increase, with the introduction of MPTs. The effect of ARV-progestin combinations on HIV-1 replication was determined and cells were infected with 40 IU of virus. TZM-bl cells were stimulated with the ligands for 24 hours and thereafter cells were infected with virus or the HEK293T media the virus was grown in, which is referred to as the virus control (VC). Data generated for HIV infection assays included experiments performed by Dr Michelle Maritz, and pooled results are depicted in figure 3.14. As shown in figure 3.13, MPA, like DEX significantly increased viral replication. The ARVs alone inhibited viral replication significantly by approximately 91% for TDF and 97% for DPV (Figure 3.14). The maximal percentage inhibition for TDF appeared to be reduced by about 10% in the presence of DEX; however, this effect was not statistically significant. Overall, the presence of 100 nM MPA or DEX had little effect on the inhibitory action of the ARVs on viral replication. Activity in the virus control would indicate that a ligand can activate the viral LTR in the absence of virus; however, neither DEX nor MPA showed this effect. These results show that 100 nM DEX and MPA alone increased viral replication, while ARVs continued to inhibit viral replication in the absence and presence of DEX and MPA.

![Graph: HIV-1 replication](image)

**Figure 3.14:** DEX and MPA increase HIV-1 replication but do not significantly affect antiviral activity of ARVs. TZM-bl cells were seeded into 96-well plates at a density of 1 x 10^4 cells/well and incubated for 24 hrs. Thereafter cells were treated with 100 nM DEX and MPA, and 1 µM TDF and DPV as well as combinations of these (DEX and TDF, DEX and DPV; MPA and TDF, MPA and DPV) and
EtOH+DMSO (0.1% v/v for each) was used as the vehicle control. Cells were incubated for 24 hrs and were then infected with 40 infectious units of HIV-1 Bal_Renilla or virus control and were incubated for 48 hrs. Thereafter, the cells were lysed and the luciferase activity was measured. In addition, a parallel experiment was done and MTT activity was measured. Luciferase activity was normalized to MTT activity. Graphs show pooled results of at least three independent experiments with each condition done in triplicate. Statistical analysis was done using a two-way ANOVA, followed by a Tukey multiple comparison post-test, comparing each treatment to all other treated conditions. Statistical significance is represented using letters where bars with the same letter are not significantly different to each other, while bars with different letters denote statistical significance.

3.6.3 DEX, MPA and DPV alone and in combination alter HIV co-receptor expression

Besides having looked at immunological genes, changes in the expression of HIV co-receptor genes, namely CCR5 and CXCR4, may provide insight into the association of DEX and MPA with increased HIV replication. In this study R5 tropic virus, which binds to CCR5, was used. To determine the effect of progestins and ARVs on HIV co-receptors and to investigate whether the increased HIV replication induced by DEX and MPA in figure 3.14 may be due to increased levels of CCR5 and CXCR4 HIV co-receptors, expression of both CCR5 and CXCR4 mRNA was investigated in TZM-bl cells using qRT-PCR. Treatment with DEX and MPA appeared to increase CCR5 mRNA expression, where DEX resulted in a significant 2.3-fold induction in CCR5 expression, while MPA resulted in a 1.8-fold induction, but did not reach significance (Figure 3.15 a). DPV, but not TDF, appeared to increase CCR5 expression. DEX also significantly upregulated CCR5 expression in the presence of TDF and DPV, while the combination of MPA with TDF and DPV showed an apparent increase, but these effects were not statistically significantly different. CXCR4 mRNA expression was not significantly altered by both DEX and MPA, in the absence and presence of TDF (Figure 3.15 b). In contrast, DPV significantly upregulated CXCR4 expression. This increase, however, was not seen in the presence of DEX and MPA, which resulted in similar levels to vehicle, suggesting that DEX and MPA may reduce the DPV-induced upregulation of CXCR4.
Figure 3.15: DEX and DPV alone and in combination differentially affect CCR5 and CXCR4 HIV co-receptor expression in TZM-bl cells. TZM-bl cells were seeded into 12-well plates at a density of 1 x 10^5 cells/well and incubated for 24 hrs. Thereafter cells were treated with 100 nM DEX and MPA, and 1 µM TDF and DPV. EtOH (0.1% v/v) was used as the vehicle control for the progestins, and DMSO (0.1% v/v) was used as the vehicle control for the ARVs. Cells were also treated with combinations of these (DEX and TDF, DEX and DPV; MPA and TDF, MPA and DPV) and EtOH+DMSO (0.1% v/v for each) was used as the vehicle control. Cells were incubated for 24 hrs and were then washed with PBS and harvested for total RNA was isolated and reverse-transcribed. Relative expression of (A) CCR5 and (B) CXCR4 mRNA was measured by qRT-PCR and normalised to GAPDH mRNA expression. Relative fold mRNA expression was normalized to the EtOH+DMSO vehicle set to 1. Graphs show pooled results of at least three independent experiments. Statistical analysis was done using a two-way ANOVA, followed by Tukey’s multiple comparison post-test, comparing each treatment to all other treated conditions. Statistical significance is denoted by * or ** to indicate P< 0.05 or P< 0.01, respectively. Statistical significance is also represented using letters where bars with the same letter are not significantly different to each other, while bars with different letters denote statistical significance.

3.7 ARVs and progestins appear to have similar effects on gene expression in cervical tissue explants as in cervical cells

3.7.1 Patient donors appear to have differing glucocorticoid and progestin sensitivities but appear to respond similarly to DPV treatment

Having established that DPV exerts gene-specific pro-inflammatory effects and that the presence of MPA can potentiate or repress the response in TZM-bl cells, the effect of DPV in combination with MPA and DEX on gene expression was determined in cervical tissue explants. HIV co-receptor expression in explants was also determined. Ectocervical explant tissue was obtained from HIV negative pre-menopausal women undergoing hysterectomies for benign reasons. Tissue was then processed and treated with ligands for 48 hrs. Viability of the tissue was determined by an MTT assay and was found to be viable after 48 hrs as determined qualitatively by a visual purple colour change in the tissue after treatment with MTT, although this was not quantified. Tissue explant cells are more difficult to lyse and thus explants were homogenized and RNA was harvested in QIAzol®. RNA was isolated using the RNeasy® Microarray Tissue Mini Kit and was run on a denaturing agarose gel. RNA integrity is shown in Figure 3.16 a, which shows successful isolation of good quality RNA, with both 28S and 18S ribosomal RNA bands intact. The ratio of these bands, however, was not exactly 2:1 as compared to the TZM-bl cell RNA in figure 3.1, but this was not surprising as tissue explant RNA is not as robust as cell line RNA due to not being completely sterile and exposed to pathogens that may degrade RNA. Thereafter cDNA was synthesized and relative mRNA expression was assessed by qRT-PCR. IL-8 and IL-6 secreted protein levels were also assessed by ELISA.
Pooled results from three donors showed that DEX and MPA did not significantly change IL-8 mRNA levels, while DPV appeared to upregulate IL-8 mRNA levels (Figure 3.16 a), as was previously seen in figure 3.4 a in TZM-bl cells but this effect was not significant, possibly due to donor to donor variation (Appendix Figure 2) and the small number of donors used for the study. The combination of MPA with DPV significantly reduced the DPV induced IL-8 mRNA response, which was different to the potentiation observed with DPV and MPA on mRNA levels in TZM-bl cells in figure 3.4 a. However, none of these mRNA changes were reflected at the protein level of IL-8, which showed no changes following treatment with DEX or MPA alone and in combination with DPV. (Figure 3.16 c).

Pooled IL-6 mRNA results from three donors showed that there were no significant changes to IL-6 mRNA expression following all treatments. DEX did appear to repress IL-6 mRNA, while MPA had no effect. DPV also appeared to increase IL-6 mRNA (Figure 3.16 d). Combinations of DEX or MPA with DPV appeared to show similar results to what was observed in TZM-bl cervical cells in figure 3.4 b. In cervical explants, DEX and MPA suppressed the DPV-induced pro-inflammatory response (Figure 3.16 d), with MPA having a greater effect than DEX, which had not previously been seen in TZM-bl cervical cells. IL-6 protein levels were highly variable but a similar trend compared to that for the mRNA results was observed (Figure 3.16 e). More repeats are required to determine whether these changes are significant in the explant model.

The classic induction of GILZ by DEX and MPA was seen, although it was not significant (Figure 3.16 f). A similar donor variation following DEX and MPA treatment was seen on GILZ mRNA expression (Appendix Figure 2 c). With regards to IL-8, IL-6, and GILZ, overall the donors appeared to respond more similarly to DPV, as DPV treatment alone did not exhibit the same large differences in donor responses as DEX and MPA did, and it also showed a similar response to what was seen in TZM-bl mRNA. These results suggest that donors may respond differently to glucocorticoids and progestins but respond more similarly to ARVs.
Figure 3.16. Effect of DPV in combination with DEX and MPA on immune function genes in cervical tissue explants. Ectocervical explant tissue samples were stimulated with 100 nM DEX and MPA, and 1 µM DPV as well as combinations of these (DEX+DPV and MPA+DPV). EtOH+DMSO (0.1% v/v for each) was used as the vehicle control and cells were incubated for 48 hours after which RNA was isolated. A) Assessment of RNA quality on a denaturing formaldehyde agarose gel. cDNA was synthesized and relative (B) IL-8, (D) IL-6 and (F) GILZ mRNA expression levels were determined by qRT-PCR and were normalized to GAPDH mRNA expression levels. To obtain relative secreted IL-8 and IL-6 protein levels supernatants were collected prior to cell harvest and cytokine protein levels for (C) IL-8 and (E) IL-6 were determined by ELISA. Relative fold change in mRNA and protein levels was determined by setting the vehicle (EtOH+DMSO) to 1. Graphs show pooled results from at least three independent experiments with each condition done in triplicate. Statistical analysis was done using a two-way ANOVA, followed by Tukey’s multiple comparison post-test, comparing each treatment to all other treated conditions. Statistical significance is denoted by * to indicate P< 0.05.

HIV co-receptor expression in cervical explants was next assessed to determine if the effects produced by DEX and MPA alone and in combination with DPV in TZM-bl cervical cells could be reflected in an ex vivo model. Overall there was large variation in the responses between donor samples most likely due to the donor biological variation. Hence no significant changes were detected in CCR5 mRNA levels following all treatments. However, there was a trend for DEX appearing to slightly increase CCR5 mRNA and similarly for DPV alone, which is consistent with the results in figure 3.15 a, but more donors are required to determine whether these changes are significant. Similar to the CCR5 mRNA levels, no significant changes were seen with CXCR4 mRNA levels in the pooled results, although DPV alone did appear to slightly increase CXCR4 mRNA levels. This would be consistent with the the result in TZM-bl cells shown in figure 3.15 b, although it was not significant.

Taken together, the cervical tissue experiments indicate that DPV appears to be able to exert gene specific proinflammatory effects and possibly alter HIV co-receptor expression in a physiologically relevant model. Although not all the responses were reflected in both the cervical cell line and the cervical explants, these results show that the in vitro cervical cell line
is necessary to investigate first for physiological responses as it can predict what the responses may be in the physiological setting.

Figure 3.17. Effect of DPV in combination with DEX and MPA on HIV co-receptor expression in cervical tissue explants. Ectocervical explant tissue samples were stimulated with 100 nM DEX and MPA, and 1 µM DPV as well as combinations of these (DEX+DPV and MPA+DPV). EtOH+DMSO (0.1% v/v for each) was used as the vehicle control and cells were incubated for 48 hours after which RNA was isolated. cDNA was synthesized and relative (A) CCR5 and (B) CXCR4 mRNA expression levels were determined by qRT-PCR and were normalized to GAPDH mRNA expression levels. Relative fold change in mRNA levels was determined by setting the vehicle (EtOH+DMSO) to 1. Graphs show pooled results from at least three independent experiments with each condition done in triplicate. Statistical analysis was done using a two-way ANOVA, followed by Tukey's multiple comparison post-test, comparing each treatment to all other treated conditions.
Chapter 4: Discussion and Conclusion

Understanding the effects of ARVs alone and in combination with progestins on immune function gene regulation is important to find the best possible ARV and progestin combination suitable for intravaginal use. Such combinations must be highly effective at protecting women from unintended pregnancy, while having minimal side effects and minimizing the risk of HIV infection and HIV transmission. Currently very few studies have looked at the effect of various progestins, ARVs, as well as progestin-ARV combinations, on the mucosal immune environment. In the present study, a select panel of progestins, the ARVs TDF and DPV alone and in combination with DEX and MPA, were investigated for their effects on the expression of immunomodulatory genes, steroid receptor regulation, and HIV replication in TZM-bl cervical epithelial cells and in ectocervical tissue explants.

4.1 DPV, unlike TDF, exerts cytotoxic and pro-inflammatory effects in the female genital tract

The ARV concentrations ranging from 0.1 nM to 10 µM, used in the dose response analysis to determine whether ARVs regulate immune function genes, are relevant. They include the nanomolar in vitro IC_{50} concentrations of ARVs (Table 2) as well as the micromolar intravaginal concentrations of ARVs in the female genital tract, currently under investigation for intravaginal delivery. Cervicovaginal fluid and vaginal tissue concentrations of DPV, used in gel and ring formulations, can range between 2 to 21 µM (Nel et al., 2009, Nel et al., 2010, Fletcher et al., 2009). These concentrations are 100 000 times higher than the in vitro IC_{50} of DPV (0.2 nM) against wild-type HIV-1. Intravaginal concentrations of TDF during ring use in women are shown to reach 180 µM, a concentration 2 000 times higher than the in vitro IC_{50} of TDF against wild-type HIV-1, which is approximately 90 nM (Keller et al., 2016, Mesquita et al., 2012).

In the present study, cell viability assays revealed that 10 µM DPV significantly reduces the viability of TZM-bl cervical cells by over 60% (Figure 3.2 a). This finding is consistent with the findings of Fletcher et al., which showed that concentrations of DPV, in the range of 10 µM to 20 µM, reduce the viability of cells by 50% in TZM-bl, PM-1 T cells and in macrophages (Fletcher et al., 2009). 1 µM DPV also resulted in a small 20% decrease in cell viability and this was not significant; however, further Western blot analysis revealed that 1 µM DPV appears to reduce total protein content in cell lysates (Figure 3.7). This result suggests that cell numbers may be reduced after treatment with 1 µM DPV, and cytotoxic effects such as cell necrosis or apoptosis may occur. Future experiments to elucidate the mechanism underlying the loss of cell viability by DPV would be beneficial. To determine whether cell death is occurring, further investigation with the use of trypan blue exclusion assays is needed.
Apoptotic events can be assessed by detection of proteins involved in apoptosis such as caspases 3 and 7. In stark contrast to DPV, TDF did not reduce TZM-bl cell viability at concentrations up to 10 µM (Figure 3.2 b) and this result is consistent with in vitro cytotoxicity studies on TDF, which have shown that concentrations as high as 100 µM TDF have no adverse effects on cervical cells (Mesquita et al., 2012).

In women, intravaginal concentrations of DPV can be as high as 20 µM or more (Table 3) and still be considered safe due to the lack of any adverse cytotoxic effects (Nel et al. 2009). This may indicate that the female genital mucosa is able to withstand high concentrations of DPV as a microbicide. Compared to the in vitro data in the present study, the different in vivo effect of DPV could be influenced by the presence of cervical mucus lining the epithelial cells as well as the vaginal microbiota, which may protect the epithelial cells from toxic concentrations of microbicides. Vaginal microbiota could rapidly metabolise the ARVs and thus make less available while the mucus could slow down the absorption of the drug in the vaginal lumen (Hussain and Ahsan 2005). In a recent study, it has been shown that non-Lactobacillus bacteria in the female genital tract metabolise tenofovir as well as reduce its microbicide efficacy (Klatt et al. 2017) in African women, further showing the ability of factors in the genital tract to affect drug availability and drug responses.

For a microbicide to be considered safe and effective, it should not be cytotoxic in cells and tissues at concentrations intended for use in humans, and should not elicit pro-inflammatory responses that can facilitate viral penetration and replication in the female genital tract mucosa (Doncel and Clark, 2010, Fichorova, 2004). The induction of the cytokines IL-8 and IL-6 has been associated with an increase in recruitment of monocytes, T cells and dendritic cells in the mucosal surfaces of the female genital tract and this can contribute to an increased risk of HIV infection (Trifonova et al., 2006). Hence inflammatory mediators such as IL-1, IL-6, IL-8, MIP-3α, and TNF-α have become candidate biomarkers for evaluating the inflammatory potential of microbicides in ex vivo models (Fichorova et al., 2001). The present study shows that 1 µM DPV exerts gene-specific pro-inflammatory effects in cervical cells and tissue. Relative mRNA expression levels of the pro-inflammatory genes IL-6 and IL-8 were consistently upregulated by DPV in TZM-bl cervical cells (Figure 3.3 a, b), while mRNA expression levels of the anti-inflammatory gene GILZ were unchanged by DPV treatment (Figure 3.3 c). In comparison, TDF at concentrations up to 10 µM had no effect on both IL-8 and IL-6 or on GILZ mRNA expression levels (Figure 3.3 b, d, f). This result is consistent with a study evaluating a 0.3% TDF gel versus a 1% TFV gel, which found that TDF does not significantly increase the inflammatory cytokines, and chemokines, MCP-1, IL-6, RANTES, IFN-γ and TNF-α (Nixon et al., 2014).
In ectocervical tissue explants the mRNA expression levels for both IL-8 and IL-6 appear to increase following DPV treatment, similar to the increase observed in TZM-bl cells (Figure 3.16 b, d). This indicates that the in vitro cervical cell line model reflects what occurs in human cervical tissue for the DPV response. These results confirm previous reports showing that changes in inflammatory responses in vivo, could be predicted by immortalized human endocervical, ectocervical and vaginal cell lines (End1/E6E7, Ect1/E6E7, and Vk2/E6E7 cells) (Fichorova et al., 2001).

Changes in IL-6 secretory cytokine levels in TZM-bl cervical cells also appear to be reflected in tissue explants (Figure 3.5 and 3.16). In ectocervical tissue explants; however, the relative IL-6 protein levels are masked by large error, which may be due to patient variation as well as other factors that may influence secreted protein levels in tissue. These include multiple proteins and factors present in human tissue, including proteolytic enzymes that could make cytokines very unstable and prone to degradation, leading to highly variable responses. Secreted IL-8 protein was not detected in medium from TZM-bl cells, possibly due to low IL-8 levels or poor sensitivity of the assay. Other possible reasons for differences in IL-8 mRNA and protein levels could be due to the kinetics involved in the production of IL-8 in the cells that may cause differences in the time you obtain detectable levels of mRNA compared to protein. In such cases detecting IL-8 protein over time would be useful to determine whether the protein is secreted at later time points. Secreted IL-8 protein was detected in ectocervical tissue explant media, but remain unchanged by DPV treatment.

A previous study showed that high concentrations of DPV (10 µM and 100 µM) induce an IL-8 pro-inflammatory response in polarized HEC-1-A endometrial cancer cells (Gali et al., 2010). Another in vitro study showed that high concentrations of DPV (100 µM) produce a small, yet non-significant increase in IL-6 and IL-8 cytokines in VK2/E6E7, CaSki and Caco-2 cells (das Neves et al., 2013). In a mouse model, DPV-loaded nanoparticles did not significantly increase IL-6, IL-8, MIP-3α and IL-1α and β (das Neves et al., 2014). Due to these pre-clinical studies, DPV has been considered to have a low inflammatory potential and has now been assessed for efficacy in two recently terminated Phase III clinical trials (Baeten et al., 2016, Nel et al., 2016). Results from the present study are consistent with previous studies and show modest increases of pro-inflammatory effects with DPV in cervical explant tissue.

It is imperative that intravaginal microbicides do not disturb the mucosal cytokine balance, and even small changes may have a large overall effect on the mucosal cytokine signaling network. A key regulator of pro-inflammatory cytokines and chemokines, such as IL-6 and IL-8, is the transcription factor NF-κB (Li and Verma, 2002). The DPV-induced pro-inflammatory effects presented in this study may be indicative of early pro-inflammatory events occurring in
response to DPV treatment that are regulated by the NF-κB inflammatory pathway. The NF-κB pathway may be induced by the cytotoxic or possibly apoptotic events occurring, which were suggested by the cell viability data in this study (Figure 3.2 a). Apoptotic and necrotic cells have previously been shown to elicit the early pro-inflammatory mediators TNF-α, MIP-1α, and MIP-2, when co-administered with the innate immune stimulator, LPS (Lucas et al., 2003). Further investigation to determine if the DPV-induced pro-inflammatory effects in TZM-bl cells occur via the NF-κB inflammatory pathway could be performed using experiments that address whether DPV influences NF-κB activation, translocation to the nucleus, and binding to promoters of cytokine genes. Detection of phosphorylated NF-κB subunits such as p65 by a Western blot, and detection of p65 recruitment to cytokine promoters by chromatin immunoprecipitation (ChIP) assays after DPV treatment, could provide useful information on the mechanisms whereby DPV may modulate the NF-κB pathway.

Steroid receptors are involved in various physiological processes including immune function, reproductive development and functioning of the central nervous system (Africander et al., 2011). Whether ARVs affect steroid receptor activity or steroid receptor levels, may indicate their potential to produce off-target, steroid hormone related side effects. The TZM-bl cell line used in the present study endogenously expresses the GR and the AR, but not the PR (Figure 3.6). In TZM-bl cells, 1 µM DPV appears to reduce total endogenous protein as it decreases GR, AR and GAPDH protein levels (Figure 3.7). This is most likely related to the cytotoxic effects associated with high concentrations of DPV (Figure 3.2 a). In contrast, 1 µM TDF has no significant effect on either AR or GR levels.

Overexpression of the GR and AR in the steroid receptor-deficient U2OS cell line allowed further investigation of the effect of ARVs on steroid receptor activity using luciferase reporter assays. Investigations into GR transactivation activity of TDF or DPV revealed that, in GR expressing cells in the absence of the agonist DEX, TDF and DPV appear to decrease GR activity, and this indicates possible ligand independent effects on GR activity (Figure 3.9). In the presence of the agonist, DPV and TDF have no significant effect on GR activity. The decrease in GR activity observed with TDF and DPV, may suggest that the ARVs have effects on GR transrepression. Further investigation of the GR-mediated repression of AP1 or NF-κB transcriptional activity, in the absence and presence of ARVs, is required, to determine if ARVs influence GR transrepression. Ligand independent effects were also observed on AR activity, since TDF and DPV appear to increase AR activity in AR-expressing cells in the absence of the agonist, MIB (Figure 3.10). In the presence of the agonist only DPV, and not TDF, significantly increased AR activity at 100 nM. This result suggests that DPV may influence the efficacy and potency of AR ligands, as confirmed by dose response analysis with MIB.
Dose response experiments using the potent AR agonist MIB in the presence and absence of 1 µM TDF or DPV showed that the presence of DPV, unlike TDF, dramatically reduces the EC_{50} of MIB, thereby increasing its potency (Figure 3.11). DPV also significantly reduces the efficacy of MIB. Previous studies have shown that a left-shifted steroid receptor ligand dose response curve can be caused by an increase in steroid receptor levels in the cells, which allows the half maximal response (EC_{50}) to be reached at lower doses of the ligand (Zhao et al., 2003). This suggests that in U2OS cells, DPV may increase the AR levels.

Results from the present study suggest that alterations in steroid receptor levels in response to ARVs may differ between cell lines since in TZM-bi cells, DPV appears to reduce endogenous protein levels, while in U20S cells, overexpressed AR protein appears to increase. Western blot analysis to confirm receptor expression was performed in the present study (Figure 3.11 a), but protein samples were only taken at one concentration of MIB. Further western blot experiments are required to assess whether AR levels are increased by DPV in the presence of increasing concentrations of MIB in U20S cells exogenously overexpressing AR.

Increased potency of AR ligands by DPV may result in increased side effects due to AR signalling. Results from this study are the first to show that DPV can alter the efficacy and potency of an AR ligand. This has implications for women on hormonal contraceptives, that would consider using DPV, as some hormonal contraceptives such as MPA and NET, have been shown to be efficacious AR agonists (Africander et al., 2014). The presence of DPV while using these contraceptives may lead to an increase in their potency for the AR and most likely an increase in off-target side effects via the AR. To further investigate steroid receptor involvement in the pro-inflammatory responses in the presence of DPV in cervical cells, gene expression changes in the absence and presence of steroid receptor antagonists, or steroid receptor knockdown, are needed.

4.2 Progestins have different effects on select immunomodulatory genes and MPA alters DPV-induced pro-inflammatory effects in the female genital tract

Having established that DPV regulates the expression of immunomodulatory genes, further investigation into whether a panel of progestins would do so on their own, and when in combination with an ARV, was performed. The progestins investigated in the study are those used extensively in areas of high HIV risk (MPA and NET), and those being investigated as candidates for use in combination with ARVs in MPTs (LNG, ETG, NES). Besides MPA and
NET, there is limited information available on the effect of other progestins on immune function gene regulation and steroid receptor involvement. The panel of progestins evaluated showed differential effects on the expression of the GR-regulated immune function genes IL-6, IL-8 and GILZ.

To confirm GR regulation of these genes in TZM-bl cervical cells, the GR agonist DEX was used, and it showed the strongest repression of IL-6 and the strongest induction of GILZ, while having no significant effect on IL-8 mRNA expression (Figure 3.3). Like DEX; MPA, LNG, NES and ETG all significantly repress IL-6 mRNA expression. All the progestins appear to increase GILZ expression, but only DEX significantly induces GILZ expression. The progestins had no significant effect on IL-8 expression, but appear to slightly increase IL-8 mRNA. Interestingly, MPA consistently appears to increase IL-8 expression in further experiments (Figure 3.4 a); however, statistical significance was not obtained. Further repeats are required, using fewer variables to investigate whether MPA induces a significant IL-8 response in TZM-bl cervical cells. MPA acted similarly to DEX showing partial glucocorticoid activity, which was consistent with studies in endocervical cells, that showed that MPA partially represses IL-6 and induces GILZ at 100 nM after 24 hrs (Govender et al., 2014). In the present study, ETG showed immunosuppressive effects on cervical cells at a lower concentration (100 nM) than what had previously been reported in PBMCs (1 µM) (Huijbregts et al., 2014). In contrast, LNG, which has previously been shown to lack immunosuppressive effects in PBMCs (Huijbregts et al., 2014), exhibited immunosuppressive effects on IL-6 mRNA expression in TZM-bl cervical cells.

Other reports show that LNG has anti-inflammatory effects on the immune response in the female genital tract. A recent study in a mouse model, showed that LNG inhibits dendritic cell activation and function as well as T cell expansion (Calla et al., 2016a). In another study, LNG-IUD use in healthy women was shown to have no significant effect on activation of T cells, dendritic cell and macrophages (Achilles et al., 2014); however, in a similar study LNG-IUD use was associated with increased concentrations of inflammatory cytokines/chemokines in the endocervix, as well as increased production of the immunosuppressive cytokine IL-10 after T cell stimulation in the endometrium (Shanmugasundaram et al., 2016). This suggests that LNG may have different effects in different compartments of the female genital tract; although, how this impacts HIV acquisition has not been established.

The present study is the first to show an immunosuppressive role for NES in cervical cells. NES significantly reduces IL-6 mRNA levels and also appears to induce GILZ mRNA levels in TZM-bl cells. Whether this effect is occurring via a GR-dependent mechanism is yet to be established. Results from the present study show that NET appears to have small
immunosuppressive effects on GR-regulated genes; however, none of these were significant. This was consistent with previous findings that NET does not act via the GR (Govender et al., 2014, Hapgood et al., 2014b).

The ability of progestins to exert differential effects on gene expression via different steroid receptors may be reflective of the ability of progestins to alter steroid receptor turnover levels. It was previously shown that potent GR ligands turn over the GR with a half-life of about 10 hrs in COS-1 cells, as assessed by a reduction in GR protein levels on a western blot (Avenant et al., 2010b). Results from the present study are consistent with this, in that the known GR ligands, DEX and MPA, show an apparent reduction in GR levels in TZM-bl cervical cells. Interestingly, NES and ETG also appear to reduce GR levels. The trend towards turnover observed with MPA, NES and ETG on GR levels, is consistent with the effects observed in the gene expression data in figure 3.1, where these ligands produced immunosuppressive effects on GILZ and IL-6 expression. NES and ETG appear to display a similar effect to DEX and MPA, which may suggest that these ligands affect GR activity.

Previous preclinical studies showed that ETG has weak GR activity, and in contrast NES was shown to have significant GR binding affinity, but no GR activity (Heikinheimo et al., 1994, Stanczyk et al., 2012). Data from the present study supports the possibility that NES and ETG may regulate the expression of IL-6 and GILZ via the GR as previously reported for DEX and MPA (Govender et al., 2014) but requires further investigation. To investigate if NES and ETG repress IL-6 via a GR-dependent mechanism, use of a GR antagonist such as RU486 or knockdown of the GR would be useful to see if the effect is lost in the absence of the GR. LNG and NET do not appear to affect GR levels. This result is consistent with the gene expression data following NET treatment, which appeared to have no GR activity; however, for LNG, this result suggests that the IL-6 repression observed with LNG treatment may not be via a GR dependent mechanism. Further GR antagonist and knockdown experiments are required to confirm this.

Among the progestins evaluated in the present study, only LNG, NET and ETG appeared to increase AR protein levels in TZM-bl cells, although this was not significant. This result correlates with previous studies which showed that LNG, NET and ETG have AR activity (Kuhl, 2011, Stanczyk, 2003). These ligands may increase AR stability, and hence prevent its rapid degradation. The rest of the panel of progestins had no overall significant effect on AR protein receptor levels. For MPA especially, this was not expected as MPA was previously shown to have AR activity (Africander et al., 2014). The presence of the GR in TZM-bl cells may play a role in this outcome. It may be that if excess GR is present, most of the MPA could bind to the
GR, which might explain why MPA appeared to turn over the GR, while having no effect on AR protein.

Data from the present study show that progestins other than MPA can also regulate immune function genes, and these may have implications for women choosing to use alternative hormonal contraceptives. Of particular interest are those that act similarly to MPA possibly via the GR, such as NES and ETG, as these may contribute to increasing the risk of HIV acquisition, by suppressing the protective properties of the local genital tract immune system. Further investigation on the effects of these progestins on the innate and early adaptive immune response is needed to fully characterize their potential as immunosuppressive agents. LNG also displayed immunosuppressive effects and these may also indirectly influence HIV acquisition. On the other hand, data from this study support NET as the better choice of progestin, as it showed no significant effect on immunomodulatory gene regulation or glucocorticoid activity.

Progestins are ideal candidates to be incorporated into MPTs and currently LNG is the first to be in development in combination with an ARV (Polis et al., 2016b). While few studies have investigated the effects of ARVs and progestins alone on immune function genes, there is no data available on the combinatorial effects of these on immune function gene regulation. To our knowledge this is the first study to investigate the effect of an ARV and a progestin combination on immunomodulatory gene expression. Findings from this study show that in TZM-bl cervical cells, MPA potentiates the DPV-induced pro-inflammatory response on IL-8 mRNA expression (Figure 3.4 a). In contrast to IL-8, MPA reduced the DPV-induced pro-inflammatory response on IL-6 mRNA and protein expression (Figure 3.4 b, 3.5). Results in this study show that individually MPA is immunosuppressive and DPV is pro-inflammatory; however, when combined, MPA dramatically changes the DPV response in a gene-specific manner. These results show that women already on MPA who may consider using DPV as a microbicide, may have further impacts on their mucosal immune environment and their risk of infection by pathogens such as HIV. Potentiation of the chemokine IL-8 may recruit more HIV target cells to the mucosa (Li et al., 2013), while a decrease in IL-6 may reduce protection against infections, which may also contribute to increased HIV risk (Hara et al., 2009).

The potentiation of the DPV-induced IL-8 response by MPA seen in TZM-bl cells is not observed at the mRNA level in the ectocervical explants. Instead, the presence of MPA causes a significant reduction in the IL-8 response (Figure 3.16), which is similar to the IL-6 result observed in explants and in TZM-bl cells. The difference in IL-8 mRNA responses seen in TZM-bl cells and ectocervical tissue explants could possibly be due to the differences in steroid receptor levels in these two systems. Previous work in our laboratory showed that
ectocervical tissue explants express both the PR and the GR (Ray, 2015). In contrast, data from the present study show that TZM-bl cells express the GR, but not the PR. This suggests that the potentiation of IL-8 mRNA with MPA and DPV does not require the PR, but may be inhibited by the presence of the PR, as seen in ectocervical tissue (Figure 3.16 b). Data from this study also revealed that the ARVs in combination with DEX, significantly reduce AR and GR levels, although the same was not seen with MPA.

The variation in the different patient donor responses to DEX and MPA was notable in ectocervical tissue explants (Appendix Figure 2), where one donor showed strong repression of IL-6 and IL-8 mRNA expression, while the other two showed weaker repression. This result demonstrates the variability of patient responses to drugs, and may be due to different glucocorticoid and progestin sensitivities in donor tissue. In ex vivo studies, patient to patient variation in gene expression is not uncommon and is influenced by factors such as differences in: endogenous hormones in tissue, genotype of the responder, age, stress, and circadian cycle that can all contribute to differences in patients response to a particular drug (Alomar, 2014, Fichorova, 2004). Overall, it seemed that all patients responded more similarly to DPV treatment than to DEX or MPA treatment.

4.3 MPA increases HIV replication and CCR5 expression in vitro but does not interfere with inhibition of viral replication by ARVs

Studies on the interaction between different types of ARVs including nelfinavir, efavirenz, or nevirapine and progestins have mostly looked at their effect on the pharmacokinetic action and contraceptive efficacy of MPA and other progestins. Previous studies indicate that in HIV-positive women, MPA levels are not reduced by ARVs and thereby suggest that ARVs have no significant effect on the efficacy of MPA (Cohn et al., 2007, Nanda et al., 2008, Watts et al., 2008).

In the present study, despite the increased viral replication observed in the presence of MPA and DEX, their combination with TDF or DPV had no significant effect on the ARVs inhibitory action (Figure 3.14). Treatment of cells with 1 µM TDF or DPV alone significantly inhibited viral replication by over 90% as expected, as concentrations as low as 10 nM for TDF and 6 nM for DPV are shown to inhibit viral replication in TZM-bl cervical cells (Fletcher et al., 2009, Mesquita et al., 2012). This study is the first to show that in an in vitro cervical cell model, MPA and DEX have no effect on the inhibitory action of DPV. For TDF, results from the present study are consistent with studies in animal models, which showed that MPA does not impact on the prophylactic activity of TDF (Radzio et al., 2014, Smith et al., 2015). From the results of the present study, it can be speculated that women on glucocorticoid therapy would be able
to benefit from PrEP, without any risk of loss of efficacy of the ARVs DPV or TDF. Although, women on MPA would benefit from the efficacy of the ARV, their altered genital immune milieu could pose a risk to their response to other infections. Further investigations with dose responses of DPV and TDF, in the presence of MPA or the other progestins, could be performed to determine whether the potency of ARV is changed in the presence of progestins.

Both MPA and DEX at 100 nM significantly increase HIV replication in TZM-bl cells (Figure 3.14) and this seems to occur with higher concentrations of virus, although this observation was only seen after two experiments (Figure 3.13). Consistent with these findings, MPA was previously shown to directly increase viral replication in vitro, in human CD3+ CD8-unstimulated PBMCs and CD4+ T cells at concentrations as low as 0.1 nM (Sampah et al., 2015). A more recent study in our laboratory has shown that 100 nM MPA increases HIV replication in a majority of cervical explants (Ray, 2015). Further investigation of this finding has shown that MPA increases HIV replication in a dose dependent manner in ectocervical explants, and in non-activated PBMCs with an EC₅₀ of 15 nM in PBMCs (Ray et al., 2017, submitted). Like MPA, DEX was shown in vitro to increase replication at a concentration of 10 nM and concentrations up to 1 µM (Ayyavoo et al., 1997, Huijbregts et al., 2013, Soudeyns and Wainberg, 1997).

A possible mechanism underlying the increased viral replication with MPA and DEX, is the regulation of expression of surface HIV co-receptors, which facilitate viral entry into a target cell. In the present study, DEX significantly increases CCR5 mRNA levels, while MPA only appears to increase CCR5 mRNA levels in TZM-bl cervical cells (Figure 3.15). In ectocervical tissue explants, DEX appears to slightly increase CCR5 mRNA levels, while MPA has no significant effect on CCR5 mRNA (Figure 3.17). R5-tropic HIV-1 was used to infect TZM-bl cells in this study and thus the increase in CCR5 is likely to be relevant, and may have contributed to the increase in HIV replication with DEX and MPA. Although X4-tropic HIV-1 was not used in the infection assays in this study, the effect of DEX and MPA on the CXCR4 mRNA levels may give an idea of the effect of these ligands on X4-tropic HIV replication. Data from the present study revealed that DEX appears to increase CXCR4 mRNA levels, while MPA has no significant effect on CXCR4 mRNA levels in TZM-bl cervical cells. In ectocervical tissue explants, DEX and MPA had no significant effect on CXCR4 mRNA.

An increase in CCR5 expression and an increase in the activation and/or frequency of CCR5+ T cells is strongly associated with an increase in HIV replication (Meditz et al., 2011). Data from the present study supports previous reports that have shown that MPA increases CCR5 expression or increases frequency of CCR5+ T cells. Chandra et al. found that 12 weeks after DMPA injection, vaginal biopsies of HIV negative women had increased frequency of CCR5+
HIV target cells (Chandra et al., 2013). Byrne et al. found that women using the progestin-only contraceptives DMPA and NET-EN had increased expression of CCR5 on CD4+ T cells. In another study by Sciaranghella et al., DMPA and LNG-IUD use was associated with an increased proportion of peripheral CCR5-expressing T cells (Sciaranghella et al., 2015). In contrast, other reports have found either no change or a decrease in CCR5 expression or CCR5+ T cell frequency with DMPA use (Michel et al., 2015, Sampah et al., 2015, Smith-McCune et al., 2017).

There are limited studies on the effects of progestins on CXCR4 expression. MPA was previously shown to have no effect on CXCR4 expression in CD3+CD8- T cells of unstimulated PBMCs (Sampah et al., 2015), while LNG was shown to increase CXCR4 expression in the female genital tract (Shanmugasundaram et al., 2016). A recent report showed that glucocorticoids induce CXCR4 expression in CD34+ cord blood hematopoietic stem cells at concentrations of 1 µM (Guo et al., 2017). In the present study, 100 nM DEX was used and this could explain why only a small increase in CXCR4 was observed in TZM-bl cells. Differences between results may also be due to different cell types being investigated.

The effect of ARVs in the absence and presence of DEX and MPA on HIV co-receptor expression was also investigated. Surprisingly, DPV treatment, which effectively inhibits R5-tropic virus (Figure 3.14), shows an apparent increase in CCR5 mRNA levels in the absence and presence of DEX and MPA in TZM-bl cells (Figure 3.15 a). DPV also appears to increase CCR5 mRNA levels in ectocervical tissue explants and the presence of DEX or MPA appears to reduce this effect (Figure 3.17 a). DPV significantly increases CXCR4 mRNA levels in TZM-bl cells (Figure 3.15 b); however, this was not observed in the presence of MPA or DEX (Figure 3.15). In ectocervical tissue explants DPV also appears to increase CXCR4 mRNA levels although this increase remains unchanged by the presence of DEX, but appears to decrease in the presence of MPA (Figure 3.17 b). Unlike DPV, TDF had no effect on HIV co-receptor expression in TZM-bl cells.

Data from the present study suggest that MPA, DEX and DPV increase HIV co-receptor expression and may in turn contribute to increased viral entry. Whether these ligands are directly involved in increasing viral entry cannot be determined from this study. The TZM-bl infection assay measures the activation of the HIV LTR found in the HIV promoter, which will depend on all the steps that occur prior to transcription of the LTR. Further investigation into viral entry using assays such as a HIV–cell fusion assays that measure the transfer of virus-encapsulated β-lactamase into the cytoplasm (Marin et al., 2015), could be used to determine if MPA, DEX or DPV directly affect viral entry. The possibility of MPA, DEX or DPV alone and in combination with each other, increasing viral entry, may pose a heightened risk to HIV
infection and transmission and although DPV still maintains anti-HIV activity, its effects in periods of non-use may cause concern for many young women who are using MPA or are on glucocorticoid therapy.

Limitations of the present study included the lack of statistical significance and low statistical power in some of the experiments, such as the gene expression experiments, investigating the effect of ARVs in combination with progestins, and the Western blot analysis for endogenous protein levels, in TZM-bl cells. These experiments had low statistical power due to the presence of large numbers of variables. Other sources of technical variation in Western blot analysis could possibly come from differences in transfer and detection of protein on the membrane, the antibody conditions or exposure times, which would further decrease the power of the analysis. Large error was also observed in the ectocervical tissue gene expression and this may have been due to patient sample variation and few biological repeats. In future experiments, the use of fewer variables, and more biological repeats may help in establishing statistical significance and increasing the statistical power of the study.

4.4 Conclusions

The current study has shown that the use of DPV may pose a risk of increasing HIV or other infections through its pro-inflammatory and cytotoxic effects, as well as its effects on HIV co-receptor expression. DPV can alter the efficacy and potency of an AR ligand which may contribute to increased side effects via the AR. Women using the microbicide alone may inadvertently increase their chances of acquiring HIV or spreading it by using the product.

DPV-induced pro-inflammatory effects in the absence and presence of MPA have implications for HIV infected women using DPV, while on the injectable DMPA, or on glucocorticoid therapy. DPV use would still prevent further replication of the virus but women may be at a greater risk of acquiring other genital tract infections such as herpes simplex virus (HSV) or human papilloma virus (HPV), as DEX or MPA in combination with DPV could alter the immune state in the female genital tract in favour of these viruses.

Although the presence of MPA or DEX has no effect on the inhibitory action of DPV, this may not mean that DPV is completely safe for use intravaginally. ARVs are known to reduce viral particles to undetectable levels in HIV infected individuals, but cannot completely get rid of all the virus due to latently infected cells. If HIV negative women using DPV for PrEP along with glucocorticoids or MPA, are exposed to HIV, viral entry could increase due to the increase in HIV co-receptor expression. Although the ARV would still inhibit the virus, risks of developing latent HIV infected reservoirs would be high. The pro-inflammatory effects of DPV and
increases in co-receptor levels may cause activation of these latent cells, and this may lead to an increase in disease transmission of the virus, especially in periods of non-use of DPV. In support, pro-inflammatory cytokines are known to be involved in HIV transmission by increasing HIV replication of latently infected cells (Alfano and Poli, 2002, Royce et al., 1997).

DPV was also shown to increase the potency of an AR ligand. This has implications for women taking hormonal contraceptives that have androgenic properties, such as MPA, NET and LNG, as the presence of DPV may exacerbate the side effects brought on by AR signalling such as weight gain, clitoral enlargement, and acne (Ghatge et al., 2005, Goodman, 2006). Thus, this study shows that DPV may not be the best ARV to be used intravaginally alone, and in combination with glucocorticoids or hormonal contraceptives.

TDF is already being used as an oral PrEP ARV in combination with FTC in many countries. The use of TDF as an intravaginal product is still under investigation in phase I trials (Bekker et al., 2016, Keller et al., 2016). From the present study, TDF’s lack of regulation of inflammatory genes, HIV co-receptor genes, steroid receptor activity or steroid receptor levels, alone or in combination with DEX or MPA, indicate that TDF may be the better choice of PrEP for women using a hormonal contraceptive, such as MPA, or women on glucocorticoid therapy, as it will not impact on the biological effects of these ligands. TDF can be used at high concentrations in the genital mucosa, while having no cytotoxic effects and women can also use TDF at the same time with hormonal contraception, without having increased chances of HIV infection through increased HIV co-receptor expression.

This study predicts that MPA and DPV combination drugs for MPT products would not be a suitable choice for women, whereas TDF in combination with a progestin, which showed little effect on immunomodulatory gene expression such as NET, may be the ideal choice for women who are at high risk of HIV infection. It is unknown whether the effects of ARVs and progestin combinations observed in this study, will occur in vivo, and thus further studies investigating these combinations and other possible ones, such as LNG and DPV combinations, is necessary in a relevant model. This can allow thorough characterisation of different combinations of ARVs and progestins to select suitable combinations that will effectively inhibit unwanted pregnancies, while also effectively reducing the risk and protecting against HIV infection.
4.5 References


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4.6 Appendix

Plasmid identity and restriction enzyme digest

To confirm plasmid integrity and identity, purified plasmids were digested using restriction enzymes as shown in Figure 3.9. All bands of expected and observed sizes are detailed in Table 5. The *EcoRI* digest of the pSV-AR plasmid confirmed the identity of the plasmid, producing expected bands of 6700 and 500 bp. The *PstI* digest of the AR plasmid produced six bands instead of four, and this was most likely due to incomplete digest of the plasmid, that produced non-specific bands. The 1906 bp band produced by the PstI digest on the pcDNA3 GR WT plasmid was not clearly visible, although it was observed to be the second band of the digest, as the third band of the digest with a size of 1791 bp was clearly visible. The double digest of the control plasmid pcDNA3 with *KpnI* and *BglII* produced bands of sizes 4500 and 800 bp and these were close to what was expected. This then confirmed that all plasmids were the correct plasmids.

Table 5: Expected and observed band sizes of restriction enzyme digest of plasmid DNA

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction Enzyme</th>
<th>Expected (bp)</th>
<th>Observed (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3 GR WT</td>
<td><em>PstI</em></td>
<td>4037,1906, 1791</td>
<td>=4000, 2000, 1800</td>
</tr>
<tr>
<td></td>
<td><em>XbaI</em></td>
<td>7734</td>
<td>=7500</td>
</tr>
<tr>
<td>pSV-AR</td>
<td><em>BamHI</em></td>
<td>7200</td>
<td>=7500</td>
</tr>
<tr>
<td></td>
<td><em>EcoRI</em></td>
<td>6700, 500</td>
<td>=6500, 500</td>
</tr>
<tr>
<td></td>
<td><em>PstI</em></td>
<td>3000, 1800, 1500, 900</td>
<td>=5000, 3000, 1800, 1500, 850, 300</td>
</tr>
<tr>
<td>pcDNA3</td>
<td><em>BglII</em></td>
<td>5446</td>
<td>=5000</td>
</tr>
<tr>
<td></td>
<td><em>KpnI</em></td>
<td>5446</td>
<td>=5000</td>
</tr>
<tr>
<td></td>
<td><em>KpnI+BglII</em></td>
<td>4646, 800</td>
<td>=4500, 800</td>
</tr>
<tr>
<td></td>
<td><em>PstI</em></td>
<td>4061, 1385</td>
<td>=4000, 1400</td>
</tr>
</tbody>
</table>
Figure 1: Plasmid restriction enzyme digest. The plasmids pcDNA3, pSV-AR and pcDNA3 GR WT were digested with 1 U of restriction enzymes for 10 mins on ice and electrophoresed on a 0.8% agarose gel at 100V for 1 hour. Bands were visualized on a SynGene, G: Box (Vacutec, England) and images were acquired using GeneSnap from SynGene, version 7.08 (SynGene, England).
Figure 2: Different donor mRNA responses to DEX and MPA in combination with DPV. Ectocervical explant tissue samples were stimulated with 100 nM DEX and MPA, and 1 µM DPV as well as combinations of these (DEX+DPV and MPA+DPV). ETOH+DMSO (0.1% v/v for each) was used as the vehicle control and cells were incubated for 48 hours after which RNA was isolated. cDNA was synthesized and relative (A) IL-6, (B) IL-8 and (C) GILZ mRNA expression levels were determined by qRT-PCR and were normalized to GAPDH mRNA expression levels. Relative fold change in mRNA and protein levels was determined by setting the vehicle (ETOH+DMSO) to 1.