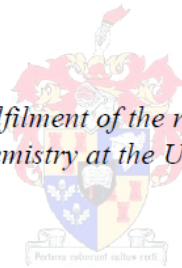# Regulation of chemokine gene expression by synthetic progestins in a human vaginal epithelial cell line.

Dewald Johan Noeth

Thesis presented in partial fulfilment of the requirements for the degree
Master of Science in Biochemistry at the University of Stellenbosch

Supervisor: Dr. Donita Africander
Co-supervisor: Prof. Janet P. Hapgood

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Declaration:

By submitting this thesis, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Dewald Johan Noeth

SUMMARY

The synthetic progestins, medroxyprogesterone acetate (MPA) and norethisterone (Net) and its derivatives (norethisterone enanthate (Net-EN) and norethisterone acetate (Net-A)), are widely used as contraceptives and in hormone replacement therapy (HRT). Several studies have indicated that synthetic progestins modulate immune function and increase the risk of sexually transmitted infections. However, little is known about the molecular mechanism of action of MPA and Net, in particular their regulation of gene expression in the female genital tract, as compared to progesterone (P4). In the first part of this thesis, the effect of P4, MPA and Net-A on the expression of the endogenous chemokine genes, macrophage inflammatory protein (MIP)-1α and MIP-1β, was investigated in a human vaginal epithelial cell line (Vk2/E6E7). Quantitative realtime PCR (QPCR) showed that both P4 and MPA upregulated the TNF-α-induced expression of MIP-1α and MIP-1β mRNA, while Net-A had no effect. Using siRNA technology, it was found that the responses to P4 and MPA on the MIP-1α gene, but not the MIP-1β gene, are mediated via the glucocorticoid receptor (GR). In the second part of the thesis, it was investigated whether the HIV-1 accessory protein, viral protein R (Vpr), could modulate the action of ligands on MIP-1α and MIP-1β gene expression. QPCR showed that Vpr abrogates the effects of P4 and MPA on the TNF-α induced expression of MIP-1α and MIP-1β. Silencing the GR with siRNA technology showed that the GR plays a role in the effect of Vpr on the P4 and MPA-induced expression of MIP-1α. Taken together, these results show that MPA and Net-A display differential effects on chemokine gene expression in a human vaginal epithelial cell line. Furthermore, this study shows that Vpr modulates the effects of MPA bound to the GR. Thus, the results of this thesis provide insight into the effect of synthetic progestins on the immune response in the vagina, and possibly how HIV-infection may alter these responses.

OPSOMMING

Die sintetiese progestiene medroksieprogesteroon asetaat (MPA) en noretisteroon (Net) en derivate daarvan (noretisteroon enantaat (Net-EN) en noretisteroon asetaat (Net-A)), word op grootskaal gebruik as voorbehoedmiddels en in hormoonvervangingsterapie (HVT). Verskeie studies het al aangedui dat sintetiese progestiene immuunfunksie moduleer en die risiko vir seksuel oordraagbare infeksies verhoog. Daar is egter min bekend oor die molekulêre meganisme van aksie van MPA en Net, in die besonder die regulering van geenuitdrukking in die vroulike geslagskanaal in vergelyking met progesteroon (P4). In die eerste deel van hierdie tesis is die effek van P4, MPA en Net-A op die uitdrukking van endogene chemokiene gene, makrofaag inflammatoriese proteïen (MIP)-1α en MIP-1β, in 'n menslike vaginale epiteel sellyn (Vk2/E6E7) bestudeer. Kwantitatiewe intydse PKR (KPKR) het getoon dat beide P4 en MPA die TNF-α-geïnduseerde uitdrukking van beide die MIP-1α en MIP-1β mRNA uitdrukking op reguleer, terwyl Net-A geen effek getoon het nie. Met die gebruik van siRNA-tegnologie is daar bevind dat die effekte van P4 en MPA, bemiddel word deur die glukokortikoïd-reseptor (GR) op MIP-1α geen uitdrukking, maar nie op MIP-1β nie. In die tweede deel van die tesis, is ondersoek of die MIV-1-bykomstigheidsproteïen, virale proteïen R (Vpr), die aksie van die ligande op MIP 1α en MIP-1β geenuitdrukking kan moduleer. KPKR toon dat Vpr die uitwerking van P4 en MPA op die TNF-α-geïnduseerde uitdrukking van MIP 1α en MIP-1β kanselleer. Die verwydering van die GR met siRNA-tegnologie toon dat die GR 'n rol in die uitwerking van Vpr op die P4 en MPA-geïnduseerde uitdrukking van MIP-1α speel. Ter samevatting: hierdie resultate toon dat MPA en Net-A differensiële uitwerkings vertoon op chemokiene geenuitdrukking in 'n menslike vaginale epiteel sellyn, en dat Vpr hierdie uitwerkings moduleer van MPA gobonde aan die GR. Die resultate van hierdie tesis werp dus lig tot die uitwerking van sintetiese progestiene op die immuunreaksie in die vagina, sowel as hoe MIV-infeksie hierdie reaksies kan verander.

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Table of Contents

ALPHABETICAL LIST OF ABBREVIATIONS

AF-1	activation function-1
AF-2	activation function-2
AIDS	Acquired immune deficiency syndrome
AP1	activator protein 1
APS	ammonium persulfate
AR	androgen receptor
ARE	androgen response element
BCG	Bacillus Calmette-Guérin
BPE	bovine pituitary extract
BSA	bovine serum albumin
CEE	conjugated equine estrogens
CFP	cyan fluorescent protein
CHD	coronary heart disease
ChIP	chromatin immunoprecipitation
Co-IP	co-immunoprecipitation
cpm	counts per minute
DBD	DNA binding domain
DEPC	diethyl pyrocarbonate
Dex	dexamethasone
DHT	dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
E.coli	*Escherichia coli*
E_2	17β-estradiol
EDTA	ethylenediaminetetra-acetic acid

EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HBD	human-β-defensin
HERS	heart and estrogen/progestin replacement study
HIV	human immunodeficiency virus
HRE	hormone response element
HRT	hormone replacement therapy
HSP	heat shock protein
HSV	herpes simplex virus
INF	interferon
KSFM	keratinocyte serum free medium
LB	Luria-Bertani
LBD	ligand binding domain
LNG	levonorgestrel
MCP	monocyte chemoattractant protein
Mib	mibolerone
MIP-1α	macrophage inflammatory protein-1α
MIP-1β	macrophage inflammatory protein-1β
MPA	medroxyprogesterone acetate

MR	mineralocorticoid receptor
MRE	mineralocorticoid response element
Mtb	*Mycobacterium tuberculosis*
Net	norethisterone
Net-A	norethisterone acetate
Net-EN	norethisterone enanthate
NFκB	nuclear factor-kappa B
P4	progesterone
PAGE	polyacrylamide gel electrophoresis
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS-BSA	phosphate buffered saline containing bovine serum albumin
PCR	polymerase chain reaction
PHA	phytohemaglutinin
PR	progesterone receptor
PRE	progesterone response element
PVDF	polyvinylidene fluoride
QPCR	quantitative realtime PCR
R5020	promegestone
RANTES	regulated upon activation, normal t-cell expressed, and secreted
SDS	sodium dodecyl sulphate
SHIV	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus
SP-1	specificity protein-1
TAT	tyrosine amino transferase
TBS	tris buffered saline
TBST	tris buffered saline containing tween

TNF-α	tumour necrosis factor-alpha
TPE	tris-phosphate-EDTA
Vpr	Viral protein R
WHO	world health organisation
YFP	yellow fluorescent protein

CHAPTER 1 : LITERATURE REVIEW

1.1 Introduction

Progestins have been classified as synthetic compounds that transform the endometrium of estrogen-primed uteri from the proliferative to the secretory phase (1). Progestins were developed to mimic the biological activity of progesterone (P4), the endogenous ovarian hormone, and are used instead of P4, as they have a longer biological half-life (2) Medroxyprogesterone acetate (MPA) and norethisterone (Net) and its derivatives, norethisterone enanthate (Net-EN) and norethisterone acetate (Net-A), are two synthetic progestins used in many countries as progestin-only injectable contraceptives and in hormone replacement therapy (HRT) (3). In South Africa, these progestins are the most commonly used female contraceptives (4).

Alarm has been raised concerning the clinical use of these progestins as studies such as the Million-women-study (5) and Women's Health Initiative (6), have shown an increase in the risk of breast cancer with the use of MPA in HRT. Additionally, the WHI (6) and the Heart and Estrogen/Progestin Replacement Study Follow-up (HERS II) (7) studies have shown that the use of MPA in HRT caused an increased incidence of cardiovascular disease and strokes. The World Health Organisation (WHO) has also reported that the use of MPA as a contraceptive could increase the risk of breast cancer (8), while the contraceptive use of both MPA and Net have been associated with increased risk of cardiovascular disease (9). Furthermore, the contraceptive use of MPA has been shown to increase HIV-1 shedding in the genital tract (10), which raises concern as to its impact on the spread of HIV-1 infection. To date very little is known about the mechanism of action of MPA and Net on local immune function in the female genital tract. A recent study by Africander *et al.* (11) showed that MPA and Net-A display differential effects on cytokine and chemokine gene

expression in human ectocervical and vaginal epithelial cells. The importance of investigating these mechanisms in the female genital tract, a site relevant to infections such as HIV, has become apparent with recent clinical evidence showing that MPA increases the risk of HIV-1 acquisition and transmission (11). Whether or not Net is associated with an increased risk of HIV-1 acquisition and transmission remain to be determined. Therefore, further research is warranted to unravel the relative effects of MPA and Net on immune function in the female genital tract. This chapter provides an overview of the synthetic progestins, MPA and Net, their mechanism of action via steroid receptors, as well as their relative effects on immune function, particularly in the female genital tract.

1.2   MPA and Net as contraceptives and in HRT

1.2.1   Structure and therapeutic applications

MPA is a 17α-hydroxyprogesterone derivative (21-carbon series steroid) containing the pregnane nucleus, and is commercially available as Depo-Provera®, Farlutal®, Provera® and Petogen®. Net and its derivatives, Net-A and Net-EN, are 19-nortestosterones containing the androstane nucleus. MPA is referred to as a true progestin, while Net is referred to as an androgenic progestin (12). The chemical structures of natural P4, and the synthetic progestins, MPA and Net, are shown in Figure 1.1 (13).

MPA and Net have many therapeutic applications in female reproductive medicine. These progestins are used primarily as injectable or oral contraceptives. Following injection, MPA is reasonably stable and is itself the active contraceptive compound (2), while Net-EN and Net-A are hydrolysed to Net and other metabolites, which together have contraceptive action (14).

A)
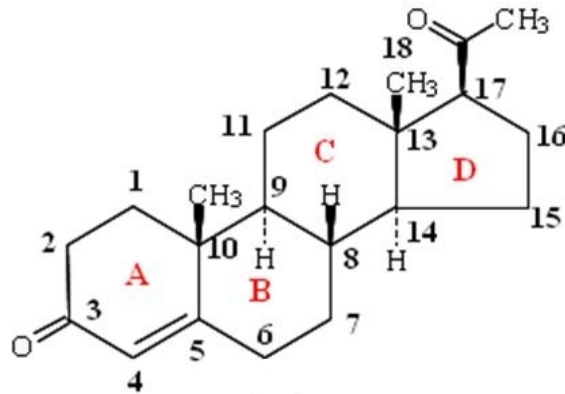
B)
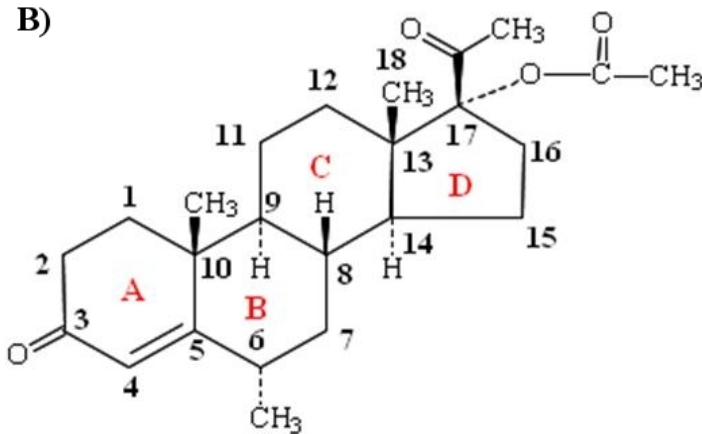
C)
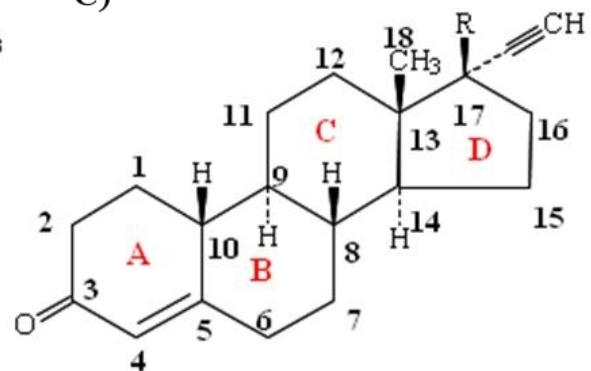
Figure 1.1: Chemical structures of (A) P4, (B) MPA and (C) Net (R=OH), Net-A (R=OCOCH₃) and Net-EN [R=OCO(CH₂)₅CH₃]. Basic ring structures are composed of 17 carbons arranged in four rings conventionally denoted by the letters A, B, C and D. Africander (13), adapted from Hapgood *et al.* (86).

MPA and Net are also used in HRT and a number of other therapeutic applications. HRT includes administration of either estrogen alone, or estrogen combined with a progestin for menopausal women with an intact uterus (15). In the latter treatment, the progestin is used to counteract the proliferative effects of estrogen on the uterine epithelium, thus preventing estrogen-induced endometrial hyperplasia (16–18). Furthermore, both MPA and Net are used in the treatment of endometriosis (19–22). MPA has also been used to treat painful disorders such as dysmenorrhea, ovulatory pain, pain associated with ovarian disease, premenstrual dysphoria, perimenopausal

symptoms and menorrhagia (20). In addition, MPA has been used at high doses for the treatment of cancer (23).

1.2.2   Side-effects of MPA and Net

As with the use of any drug, side-effects have been reported with the clinical use of MPA and Net. For example, the use of MPA as a contraceptive increases the risk of breast cancer, while the use of MPA in HRT has been associated with an increased risk for breast cancer, cardiovascular disease and strokes (5,6,9,24,25). Similarly, the use of Net as a contraceptive, has been associated with an increase in the risk of cardiovascular disease (5), while Net used in HRT has been shown to increase the risk of breast cancer (9). The clinical use of the progestins has also previously been shown to exert effects on the immune system. For example, a study in malnourished Bangladeshi women using either MPA or Net-EN as injectable contraceptive, showed that their cell-mediated immune function was compromised, by an impairment of the T cell immune response (26). Similarly, MPA has also been shown to affect various immune cell subsets in menopausal women using MPA in HRT, such as decreasing $CD4^+CD45RO^+$, $CD56^+$ and $CD8^+CD11b^+$ cells, thereby reducing the immune response during an infection (17). Furthermore, Wakatsuki *et al.* (27) showed that the use of MPA in HRT decreases plasma inflammatory markers in postmenopausal women.

A number of studies have reported the effects of MPA and/or Net on cytokine/chemokine expression or secretion (11,23,28–37). Cytokines and chemokines (chemotactic cytokines) are immune regulating proteins which elicit an immune response by activating and recruiting immune cells to the site of inflammation (38). Yamashita *et al.* (23) showed that serum levels of interleukin (IL)-6, which is predominantly pro-inflammatory, are decreased in metastatic breast carcinomas, with orally administered MPA. Similarly, MPA has been shown to repress IL-6 secretion *in vitro*, in a breast cancer cell line (KPL-40), an anaplastic thyroid cancer cell line (KTC-2), as well as a mouse

fibroblast cell line (L929sA) (29–31). MPA has also been shown to decrease the levels of other pro-inflammatory cytokines such as IL-1α and IL-12p40, as well as the anti-inflammatory cytokines IL-10 and IL-13, in peripheral blood mononuclear cells (PBMCs), in response to Bacillus Calmette-Guérin (BCG) induction (28). A study in postmenopausal women using combined HRT treatment of conjugated equine estrogens (CEE) and MPA, showed a significant decrease in the production of the pro-inflammatory cytokines, IL-2 and interferon (IFN)-γ, in phytohemaglutinin (PHA)-stimulated PBMCs, while the levels of IL-4, an anti-inflammatory cytokine, remained unchanged (32). Similarly, when used in the treatment of hormone related cancers, MPA decreased the expression of pro-inflammatory tumour necrosis factor-alpha (TNF-α), IL-1β and IL-6 in PHA-stimulated PBMCs (33).

Very few studies have addressed the effect of Net, Net-EN or Net-A on immune function. Koubovec *et al.* (34) used promoter-reporter assays to show that MPA fully repressed an IL-8 promoter reporter gene, while Net-A only repressed the reporter gene by 22% at saturating concentrations. Similar differential effects by MPA and Net on immune markers were also shown in the MCF-7 human breast cancer cell line, where MPA significantly downregulated TNF-α stimulated monocyte chemoattractant protein (MCP)-1 production, while Net upregulated the production (35). In contrast, Net-A, in combination with estradiol (E_2), has been shown to significantly decrease circulating serum levels of MCP-1 and regulated-upon-activation-normal-T-cell-expressed-and-secreted (RANTES) in postmenopausal women (36). In agreement with the above studies showing differential effects of MPA and Net-A on chemokine gene expression or secretion, Africander *et al.* (11) recently showed that MPA repressed RANTES gene expression in a human ectocervical cell line, while Net-A had no effect. Further studies are needed to understand the mechanism and functional consequence of the differential regulation of gene expression by MPA and Net.

These immunosuppressive effects, by MPA and/or Net, may have serious implications for infections such as *Mycobacterium tuberculosis* (Mtb) and HIV. For example, TNF-α, which was shown to be repressed by MPA (33), is required for the host defense against Mtb (37). Thus, women using MPA may be unable to elicit an optimal immune response when infected with Mtb. Furthermore, a recent clinical study showed that the use of MPA as a contraceptive increases the risk of HIV-1 infection two-fold (39). A number of studies have investigated the use of MPA as a risk factor for HIV-1 infection. For example two studies have shown that the contraceptive use of MPA increase the HIV-1 shedding in the genital tract (10,40). Mostad *et al.* (41), also showed that MPA increased herpes simplex virus (HSV) shedding in the cervix of HIV-infected women. The use of MPA during early HIV-1-infection has also been shown to increases the viral load (42) and the seroprevalence of HIV-1 (43). The seroprevalence refers to the number of people in a population who test positive for HIV-1 based on blood specimens.

A recent study has indicated that young women are at higher risk of HIV-1 acquisition than older women (44). However, Morrison *et al.* (45) showed that the contraceptive use of MPA was not associated with HIV-1 disease progression.

Interestingly, in animal studies, MPA has been used to increase simian immunodeficiency virus (SIV) susceptibility in macaques (46), and was shown to change the local immune response to genital HSV-2 (47). A study in simian-human immunodeficiency virus (SHIV) infected rhesus macaques administered MPA showed increased susceptibility to vaginal SHIV transmission and a decrease in the antiviral cellular immune response which indicates an immune-based rather than transmission-based mechanism (48). Together the above studies suggest that MPA can exert both systemic and local immunosuppressive side-effects *in vivo*.

In contrast to what is known for MPA, the effect of Net on HIV-1 infectability and disease

progression remains to be determined. Two studies in a cohort of South African women showed that the use of Net-EN as injectable contraceptive is not associated with increased risk of HIV-1 acquisition (49,50). However, these studies also suggest that MPA does not increase the risk of HIV infectability which contradicts many of the above mentioned studies.

For heterosexual transmission of HIV-1, the female genital tract, particularly the vagina, is the first point of entry. The precise mechanism of infectability in the female genital tract, and the factors that may influence it such as effects of endogenous hormones or hormonal contraception on local immunity, are unclear. Further investigation into these mechanisms is warranted, especially for developing effective microbicides.

1.3   Immunity in the female genital tract and the role of hormones

The female genital tract consists of two compartments, which are structurally divided by the cervix (51). The upper genital tract consists mainly of the ovaries, the fallopian tubes, the uterus and the endocervix, while the lower female genital tract consists of the ectocervix and the vagina (51). Figure 1.2 depicts a basic illustration of the compartments of the female genital tract (51,52). Epithelial cells lining the mucosal surface of the lower female genital tract, particularly the vagina, are the first point of entry for pathogens such as HIV. Regulation of the immune response in the female genital tract plays a crucial role in protection against pathogens. Initially it was believed that, on its own, the vagina was not part of the mucosal immune system (53). However, it has been shown that the vaginal mucosa of mice contains large numbers of epithelial cells, dendritic-like Langerhans cells, macrophages and T cells (54), and that all these vaginal cells play an important role in eliciting an immune response. Similarly, epithelial cells of the vagina are increasingly recognized as playing an active role in both the innate and acquired immune function by producing

pro- and anti-inflammatory cytokines/chemokines such as IL-6, IL-8, RANTES, macrophage inflammatory protein (MIP)-1α, MIP-1β, and IL-10 (55,56).
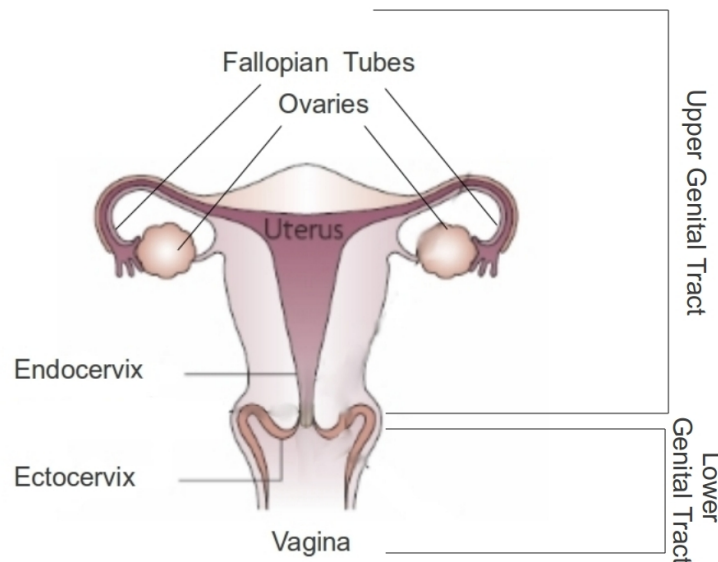
Figure 1.2: Basic illustration of the female reproductive tract. Figure adapted from Sherwood (51) and Hladik and McElrath (52).

Since cytokines/chemokines can induce inflammation, as possible determinants of susceptibility to HIV-1 infection, these immune regulators play an important role in resistance and susceptibility to infections in the vaginal mucosa. Increased levels of the chemokines RANTES, MIP-1α and MIP-1β in cervicovaginal secretions have been associated with increased HIV-1 infection (57). The susceptibility to HIV-1 infection in women may be better understood if the factors that may affect the immune response in the vagina are known. For example, sex hormones have been shown to influence susceptibility and disease predisposition for many genital tract infections (58). Recent studies have shown that during the secretory stage of the normal menstrual cycle, the female genital tract is more susceptible to viral infections due to an increase in progesterone (P4) and estradiol levels (59). Changes in the hormone levels in the female genital tact have a substantial effect on the adaptive and the innate immune function in the female genital tract, such as changes in the migration of macrophages, B cells and neutrophils, as well as changes in the expression of cytokines and chemokines (59–61).

It has previously been shown that P4 suppressed the mRNA expression of the pro-inflammatory chemokine IL-8 in the endometrium (62). More recently, P4 has been shown to decrease the production of human-β-defensin (HBD)-2 in vaginal epithelial cells (63). On the other hand, P4 significantly increased the secretion of MIP-1β by the endometrium (64). Similarly, Africander *et al.* (11) have recently shown that P4 upregulates the expression of IL-6, IL-8 and RANTES in human ectocervical and vaginal epithelial cell lines. In the same study, MPA significantly upregulated the TNF-α induced expression of IL-8, while Net-A showed a slight non-significant upregulation in the Ect1/E6E7 cells. Interestingly, a significant repression of TNF-α stimulated RANTES expression by MPA was shown in the Ect1/E6E7 cell line, while Net-A had no effect (11). Both MPA and Net-A had no effect on IL-6 gene expression in either the Vk2/E6E7 or the Ect1/E6E7 cell lines (11). Another recent study in the human endocervical epithelial cell line, End1/E6E7 (65), showed that MPA and Net-A upregulated IL-8, IL-6 and RANTES expression to diffent extents, while P4 significantly upregulated only IL-6 expression.

Taken together, the above studies show that cytokine/chemokine genes in the cervicovaginal environment are regulated in a ligand and cell specific manner by P4, MPA and Net-A. As these hormones exert their biological effects via binding to steroid receptors, it is likely that the differential effects on cytokine/chemokine gene regulation are due to these hormones acting via different steroid receptors.

Considering the above, it is clear that more research is needed on the effects of MPA and Net-A on the other genes involved in immunity in the female genital tract.

1.4   Mechanism of action of MPA and Net via steroid receptors

MPA and Net were designed to mimic the biological activity of P4, via binding to the progesterone receptor (PR). However, it is known that MPA and Net can also bind to the glucocorticoid receptor (GR), androgen receptor (AR) and the mineralocorticoid receptor (MR), in this way regulating many different genes. For example, Zhao *et al.* showed that the repression of RANTES by MPA in human endometrial stromal cells, was mediated via the PR (66). In contrast, Africander *et al.* showed that the AR mediates the repression of RANTES mRNA expression by MPA in a human ectocervical cell line (11). Furthermore, the upregulation of GILZ (glucocorticoid-induced leucine zipper) mRNA expression in both a human osteosarcoma cell line (U2OS) (67) and a human lung epithelial cell line (A549) (68), was shown to be mediated via the GR. The above studies confirm that MPA can regulate genes via different steroid receptors.

Thus, the physiological effects of MPA and Net on target genes may be influenced by steroid receptor content, as well as the cell type.

1.4.1   General mechanism of action of steroid receptors

Steroid receptors, a subfamily of the nuclear receptor superfamily, are hormone-activated transcription factors that can either activate or repress gene expression. These receptors are a highly conserved family of proteins and include the PR, AR, GR, MR and estrogen receptor (ER). A schematic presentation of the steroid receptor domain organization is shown in Figure 1.3. The central zinc-finger DNA-binding domain (DBD) is a highly conserved domain, while the C-terminal ligand-binding domain (LBD) is only moderately conserved. The N-terminal domain contains a highly variable transcriptional activation function-1 domain (AF-1) and can vary in

length and sequence between the steroid receptors (69). The specificity of a steroid receptor's response has been suggested to be determined by this highly variable region (69). Furthermore, the N-terminal domain has been shown to influence the co-factor- and LBD interaction as well as protein-protein interaction with other transcription factors (69,70). The AF-2 domain is located within the LBD and is a docking site for co-factors.
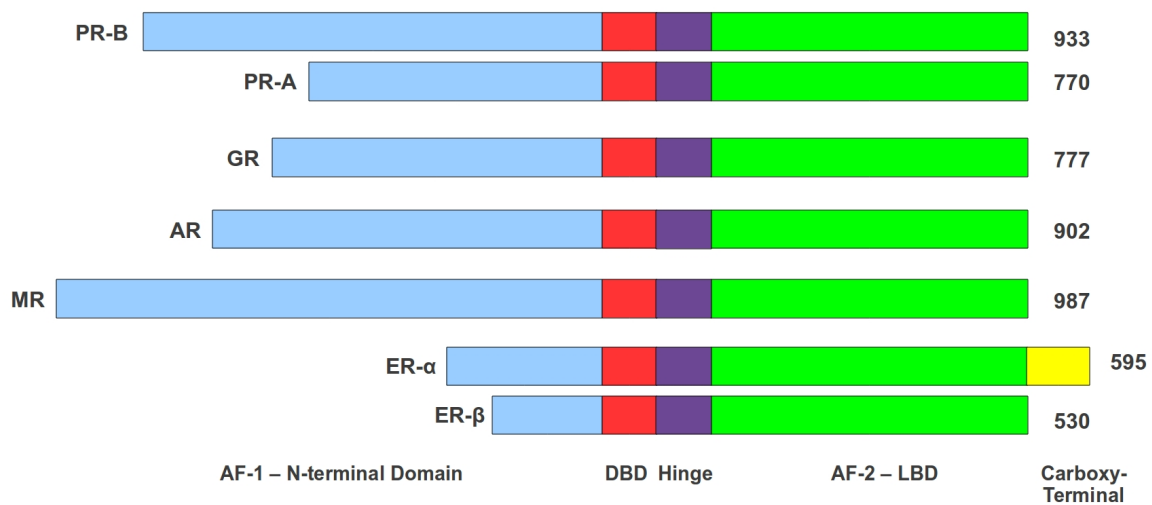
Figure 1.3: Schematic presentation of the structural- and functional domains of steroid receptors. Steroid receptors are composed of an N-terminal domain containing the AF-1 transactivation domain (blue boxes), a highly conserved DNA binding domain (DBD) (red boxes), a hinge region (purple boxes), and the carboxy-terminal ligand binding domain (LBD) containing the AF-2 transactivation region (green boxes). ER-α contains an additional carboxy-terminal domain (yellow box). The numbers on the right represent the number of amino acids in the steroid receptor. Figure adapted from Africander *et al.* (81)

When no hormone is present, the GR, AR and MR are mostly found in the cytoplasm, while the PR and ER are mostly found in the nucleus (71), complexed with heat shock protein (HSP)-90, HSP-70, immunophillins and other factors (72). An illustration of the general mechanism of action of steroid receptors is shown in Figure 1.4. When a lipophilic hormone diffuses across the plasma membrane (73), it binds to the steroid receptor which undergoes a conformational change, causing the steroid receptor to dissociate from HSP and translocate to the nucleus (71). The hormone-bound receptor binds as a dimer to specific sequences called hormone response elements (HREs), thereby activating transcription. This process is referred to as transactivation. Due to the DBD being highly conserved, most of the steroid receptors can bind to the same HRE (reviewed in 74). For example,

the PR, GR, AR and MR can all bind the same HRE, which would be termed the progesterone response element (PRE), the glucocorticoid response element (GRE), androgen response element (ARE) and mineralocorticoid response element (MRE), respectively depending on which steroid receptor binds to the response element (reviewed in 75). In contrast, steroid receptors can repress transcription by the hormone-bound receptor monomer tethering to another transcription factor such as nuclear factor-kappa B (NFκB) or activator protein 1 (AP1), in a process known as transrepression (76). The GR has been associated with the repression of many genes involved in immune responses such as cytokines and chemokines (76). Although the PR, AR and the MR have been previously shown to antagonise NFκB (77–80), much less is known about the mechanism by which the steroid receptors repress gene expression.
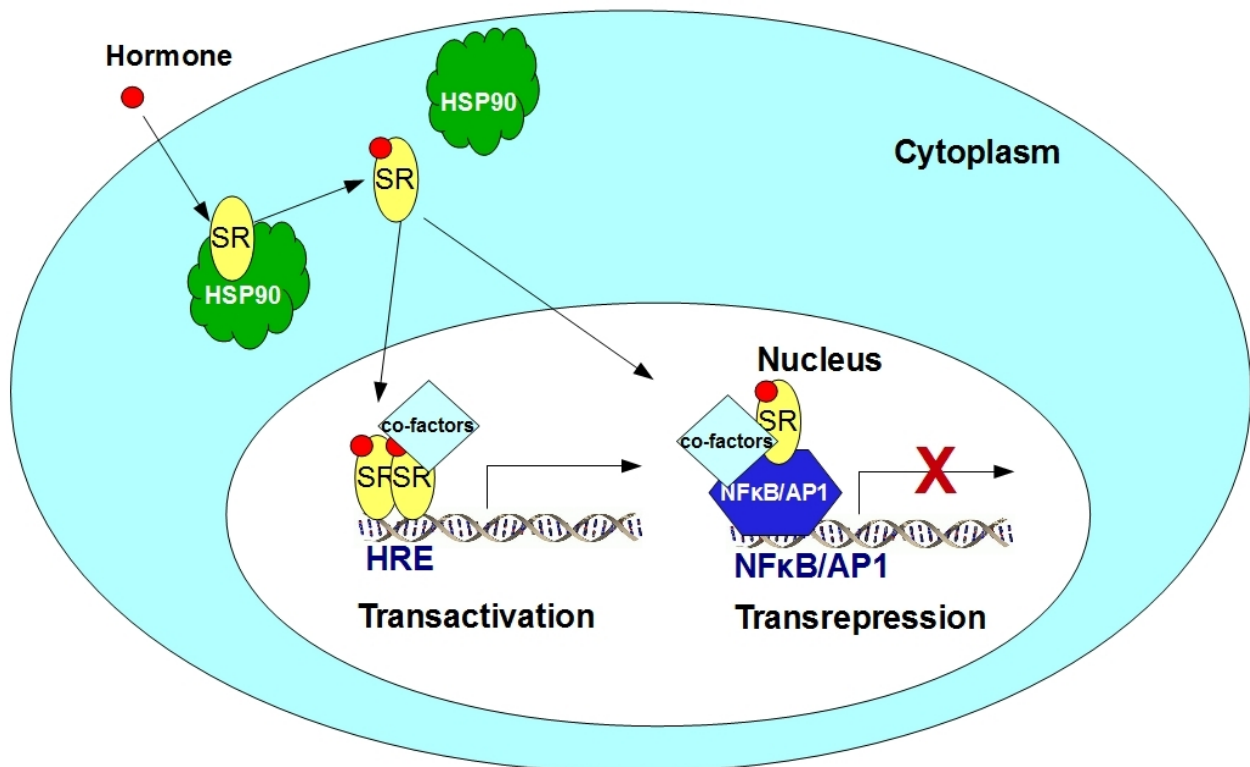

Figure 1.4: An illustration of the general mechanism of action of steroid receptors. Abbreviations: SR, steroid receptor; HSP90, heat shock protein-90; NFκB, Nuclear factor-kappaB; AP1, activator protein 1; HRE, hormone response element. Figure adapted from Africander *et al.* (81)

1.4.2 The effects of MPA and Net on target genes via different steroid receptors

As already mentioned, MPA and Net can bind to the PR, GR, AR and MR, and the progestogenic activity of MPA and Net, like P4, is elicited via the PR (reviewed in 81). In terms of the relative binding affinity for the PR, MPA has a three-fold higher relative binding affinity than P4, while Net has a 1.3 times higher relative binding affinity (82). The K_d of Dex for the GR was shown to be 4.2 nM and the K_i of P4, MPA and Net-A was found to be 215 nM, 10.8 nM and 270 nM respectively (34). The relative binding affinities of MPA and Net for the AR are very similar and relatively low (82–84). Both MPA and Net bind with a very low affinity to the MR and most studies report that it does not bind to the ER (81,82,84,85). However, binding alone does not indicate the biological activity of a hormone (34,86). For example, although MPA binds the PR with higher affinity than P4 (82), its ability to activate the PR is not always better and appears to be dependent on the cell system under investigation (reviewed in 86,87). One of the reasons for cell-specific effects by hormones such as MPA and Net-A, may be due to the steroid receptor content of the cells. Thus cellular response may be affected by type and levels of steroid receptors available, the nature and level of different isoforms, as well as the activation of different signalling pathways (86). P4, MPA and Net have been shown to bind the AR, but while MPA and Net have androgenic activity, P4 display anti-androgenic activity (82–84). Similarly, P4, MPA and Net bind to the MR, but MPA and Net-A do not display any agonist or antagonist activity, while P4 has potent antagonist activity (88). Furthermore, MPA, but not Net-A, has been shown to have partial glucocorticoid agonist activity via the GR (34) In short, the binding affinities and transcriptional activities of MPA and Net via the PR, AR and MR are similar, while their glucocorticoid-like properties differ. Due to these glucocorticoid-like properties of MPA, and given that glucocorticoids impact on the inflammatory and immune response, MPA may exhibit side-effects via the GR by acting on genes involved in immune function, such as cytokine and chemokine genes.

A number of studies have clearly indicated glucocorticoid-like effects of MPA on immune function. Kontula *et al.* (89) showed that in human mononuclear lymphocyte cells MPA induced glucocorticoid-like inhibition of the proliferative responses to T-cell mitogens. Interestingly this study also showed that Net did not display any glucocorticoid-like activity (89). Bamberger *et al.* (90) showed that MPA suppressed IL-2 transcription to the same extent as Dex in normal human lymphocytes, suggesting a role for the GR. Similarly, Kurebayashi *et al.* (91) have shown that the GR mediates the suppression of IL-6 mRNA by MPA in a thyroid cancer cell line, KTC-2, while Koubovec *et al.* (31) showed that the GR partly mediates the repression of IL-6 and IL-8 promoter-reporter constructs in both L929sA mouse fibroblast cells and African green-monkey kidney fibroblast (COS-1) cells (31). These glucocorticoid-like immunosuppressive effects of MPA via the GR, raises the possibility that MPA may increase susceptibility to infections such as HIV-1.

1.5   The GR and the HIV-1 accessory protein viral protein R (Vpr)

There is evidence in the literature suggesting that the GR could be an important protein used by HIV-1 for its pathogenic success (92–100). At least two studies have indicated that endogenous genes involved in immune function, and modulated by HIV-1 infection, are targets for the GR (92,93). The GR has also been shown to be involved in HIV-1 replication and transcription of viral proteins from the long terminal repeat (LTR) of the HIV-1 promoter (95–97,101). The HIV-1 LTR contains GR binding sites in its promoter, which may explain the involvement of the GR in the replication of HIV-1 (96,98–100,102). Furthermore, Mirani *et al.* (92) showed that the HIV-1 accessory protein, Vpr, enhanced the suppressive effects of the ligand-activated GR on the secretion of the pro-inflammatory cytokine IL-12. Decrease in expression of IL-12 in human blood cells is an indication of disease progression to AIDS (94).

Vpr is a 15 kDa protein and one of only 15 proteins encoded by the HIV-1 virus (103). Vpr is packaged within the viral particle and may play a role in the early stages of viral replication (104–106,196). Vpr has diverse functions which include host gene regulation, cell-cycle arrest and apoptosis (94,107–110). Furthermore, Vpr has been found in the blood plasma of HIV-1 infected individuals and is able to penetrate cell membranes (92,111). The possibility thus exists that Vpr may exert its effects on uninfected cells (94), such as vaginal epithelial cells.

In addition to the study by Mirani and co-workers (92), many other studies have indicated that the GR function can be modulated by Vpr (93,95,112,113). For example, Mirani *et al.* (92) showed that the GR antagonist, RU486, inhibited the Vpr induced down regulation of IL-12, suggesting that the effects of Vpr is GR-dependent. Vpr also has been shown to act as a co-activator of the GR *in vitro* by interacting with the GR at its co-activator motif (LXXLL) found in the AF-2 domain (97,114,115). A number of studies show that the GR and Vpr co-operate to regulate transcription of host genes containing GRE and NFκB regulatory elements in their promoters. Vpr has been shown to increase transcription of host genes containing GREs by acting as a co-activator of the liganded-GR (92,93,95,97,101) while Vpr can repress cytokine genes in a NFκB-dependent manner (108,116).

Vpr, in the absence of GR ligand, can also regulate host genes. Janket *et al.* (110) used micro array assays to show that Vpr downregulates many host genes, including those responsible for DNA repair, cell-cycle/proliferation regulators, tumour associated receptors and immune activation markers such as interferon regulatory factors 5 and 7. Similarly, Muthumani *et al.* (117) have previously shown that Vpr downregulates the secretion of the chemokines MIP-1α, MIP-1β and RANTES in human primary lymphocytes and macrophages. In addition, Vpr has also been shown to downregulate IL-12 and TNF-α, as well as NFκB (reviewed in 118).

As MPA, and possibly Net, can exert glucocorticoid immunosuppressive effects on cytokine and chemokine genes via the GR, the question is raised as to whether Vpr could modulate these effects of MPA and Net on target genes in a physiologically relevant cell system.

1.6 Aims of thesis

The primary aim of this study was to investigate the regulation of the genes encoding for the chemokines, MIP-1α and MIP-1β, by P4, MPA and Net-A in a human vaginal epithelial cell line. Furthermore, it was investigated whether the GR mediates any of the effects, and whether the HIV-1 accessory protein Vpr, could modulate these effects.

The following stepwise experimental approach was followed:

1. An investigation of the regulation of endogenous chemokine gene expression.

    The mRNA expression of TNFα-induced MIP-1α and MIP-1β in response to the endogenous female hormone, P4, and the two synthetic progestins, MPA and Net-A, was investigated in an immortalized human vaginal epithelial cell line (Vk2/E6E7).

2. Investigating the mechanism of gene regulation via the GR.

    The expression of TNFα-induced MIP-1α and MIP-1β in the presence of the progestins was re-evaluated when GR protein levels were decreased using siRNA technology.

3. Investigating whether the HIV-1 accessory protein Vpr modulates the progestin-induced regulation of MIP-1α and MIP-1β gene expression.

The mRNA expression of TNFα-induced MIP-1α and MIP-1β in response to P4, MPA and Net-A, was investigated in the Vk2/E6E7 cell line in the absence and presence of overexpressed Vpr.

4. Investigating whether the effects of Vpr on the progestin-induced regulation of MIP-1α gene expression are GR-dependent.

The mRNA expression of TNFα-induced MIP-1α in response to P4, MPA and Net-A, in the Vk2/E6E7 cell line in the absence and presence of overexpressed Vpr, was re-evaluated when GR protein levels were decreased using siRNA technology.

5. An investigation of whether Vpr could modulate potency and efficacy of ligands via the GR.

Promoter reporter assays in the COS-1 monkey kidney cell line was performed to investigate whether Vpr could modulate the potencies and efficacies of dexamethasone, a GR-specific agonist, and MPA, a partial agonist for the GR, via overexpressed GR in the absence and presence of overexpressed Vpr.

CHAPTER 2 : MATERIALS AND METHODS

2.1   Test compounds

Progesterone (P4) [4-pregnene-3,20-dione], medroxyprogesterone acetate (MPA) [6α-methyl-17α-hydroxy-progesterone acetate], norethisterone acetate (Net-A) [17α-ethynyl-19-nortestosterone 17β-acetate], dexamethasone (Dex) [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione] and 17β-estradiol (E$_2$) [17β-estra-1,3,5(10)-triene-3,17-diol] were purchased from Sigma-Aldrich Pty Ltd., Kempton Park, South Africa. [^{3}H]-E$_2$ (110 Ci/mmol) was purchased from AEC-Amersham, Cape Town, South Africa. Stock aliquots of 0.1 μg/ml recombinant mouse tumour necrosis factor-alpha (TNF-α) (Sigma-Aldrich Pty Ltd., Kempton Park, South Africa) were diluted in sterile H$_2$O and stored at -20ºC. The working stock solution (20 mg/ml) diluted in keratinocyte serum free medium (KSFM) (GibcoBRL, Paisley, Scotland) was also kept at -20ºC.

2.2   Cell culture

Human vaginal epithelial (Vk2/E6E7) cells, as well as African green-monkey kidney fibroblast (COS-1) cells were obtained from the American Type Culture Collection (ATCC, Virginia, United States of America). Vk2/E6E7 cells were maintained in 75-cm^2 culture flasks (Greiner Bio-One International, Kremsmuenster, Austria) in KSFM supplemented with 50 μg/ml bovine pituitary extract (BPE), 0.1 ng/ml epidermal growth factor (EGF) (GibcoBRL, Paisley, Scotland), 50 IU/ml penicillin, 50 μg/ml streptomycin (GibcoBRL, Paisley, Scotland), and CaCl$_2$ to a final concentration of 0.4 mM, hereafter referred to as complete KSFM medium. The COS-1 cells were maintained in 75 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM) from Sigma-Aldrich Pty Ltd., Kempton Park, South Africa, supplemented with 10% (v/v) fetal calf serum (FCS) (Highveld

Biologicals, Lyndhurst, South Africa), and 50 IU/ml penicillin and 50 µg/ml streptomycin, hereafter referred to as complete DMEM. Cells were grown at 37ºC in an atmosphere of 90% humidity and 5% CO_2. Both cell cultures were passaged twice weekly with 0.25% trypsin and 0.1% EDTA in calcium- and magnesium-free phosphate-buffered saline (PBS). To neutralize the activity of the trypsin, 10 ml neutralisation medium [DMEM containing Ham's F-12 Nutrient Mixture (1:1) (GibcoBRL, Paisley, Scotland), 50 IU/ml penicillin and 50 µg/ml streptomycin and 10% (v/v) fetal calf serum)] was added to the Vk2/E6E7 cells. For the COS-1 cell line, trypsin's activity was neutralised using complete DMEM. All cell lines were regularly tested for mycoplasma, and only mycoplasma negative cell lines were used in experiments.

2.3   Plasmid DNA preparations

Heat shock competent *Escherichia coli* (*E.coli)* cells were made using 0.1 M $CaCl_2$ and stored in 15% (v/v) glycerol at -80ºC. Cells were transformed by adding 2 µg plasmid DNA to 50 µl competent cells. Next, cells were placed on ice for 25 minutes followed by 52ºC for 1 minute and then returned to ice for 2 minutes. Thereafter, 1 ml Luria-Bertani (LB) medium (Addendum D) was added and the cells were incubated for 1 hour at 37ºC. Cells were then grown on a LB agar plate containing 50 µg/ml ampicillin (Addendum D) for 16 hours at 37ºC. A single colony was picked and grown for 16 hours at 37ºC in 250 ml LB medium containing 50 µg/ml ampicillin. All plasmids used during this study contain an ampicillin-resistance gene. Cultures were centrifuged at 5000 x g and the supernatant discarded. Plasmid purification from bacterial constructs was performed at room temperature using the PureYield™ Plasmid Maxiprep System (Promega, Madison, United States of America) according to the manufacturer's instructions. Briefly, the bacterial pellets were resuspended in 12 ml Cell Resuspension Solution after which the cells were lysed with 12 ml Cell Lysis Solution, followed by inverting the tube five times and incubation at room temperature for 3

minutes. Lysis was stopped by adding 12 ml Neutralization Solution and inverting the solution 10-15 times. The lysate was centrifuged at 14000 x g for 20 minutes. The plasmid DNA was purified on two columns, first a PureYield™ clearing column and then a PureYield™ maxi binding column. The columns were set up on a vacuum manifold so that the eluate of the PureYield™ clearing column was eluted into the PureYield™ maxi binding column. The lysate was decanted into the PureYield™ clearing column and maximum vacuum was applied. After the lysate had passed through the PureYield™ clearing column, the vacuum was released and the PureYield™ clearing column removed. The PureYield™ maxi binding column was washed with 5 ml Endotoxin Removing Buffer and 20 ml Column Wash using maximum vacuum. The column was dried for 5 minutes using maximum vacuum. Nuclease-free water (1.5 ml) was added to the spin column and centrifuged in a swing bucket rotor at 2000 x g for 5 minutes. The eluate containing the plasmid DNA was collected and the concentration determined on a NanoDrop 1000 (Thermo Fisher Scientific, Johannesburg, South Africa). Plasmid DNA size and integrity was analysed by restriction enzyme digests and agarose gel electrophoresis. A list of all plasmids, as well as the plasmid maps used in this study can be found in Addendum A.

2.4   Promoter reporter assays

2.4.1   Transactivation assays in the Vk2/E6E7 cell line

Vk2/E6E7 cells were seeded in 24-well plates at a concentration of 5×10^4 cells per well. The next day, cells were transiently transfected using FuGENE6 transfection reagent (Roche Diagnostics Pty. Ltd., Randburg, South Africa) according to the manufacturer's instructions. The cells were transfected with 30 ng pGL2-basic empty vector, 300 ng pTAT-GRE-E1b-luc, driven by the E1b promoter that contains two copies of the rat tyrosine amino transferase (TAT)-GRE, and 30 ng β-galactosidase (pSV-βgal). After 24 hours, cells were treated with 0.1% (v/v) EtOH or 10 μM test

compound in supplement-free KSFM. Cells were then lysed with 50 μl passive lysis buffer (Addendum D) and stored at -20ºC. The protein concentration was determined using the standard Bradford assay method (119) and 10 μl of the samples were used to determine the luciferase activity using the Promega luciferase assay system (Promega, Madison, United States of America), and a Veritas™ microplate luminometer.

2.4.2   *Transactivation assays in the COS-1 cell line*

COS-1 cells were seeded in 10 cm plates at a concentration of $2x10^6$ cells per well. Twenty-four hours after plating, cells were transiently transfected using FuGENE6 transfection reagent according to the manufacturer's instructions. The cells were transfected with 900 ng human GRα (pRS-hGRα) or pGL2-basic empty vector, 9000 ng pTAT-GRE-E1b-luc, 900 ng pSV-βgal and either 900 ng HIV-1 Vpr (pCMV4-3HA·Vpr) or pGL2-Basic empty vector. Cells were incubated for 24 hours and subsequently replated into 96-well plates at a concentration of $1x10^4$ cells per well. The next day the cells were incubated for 24 hours with either 0.1% (v/v) EtOH or increasing concentrations of test compounds Thereafter, the cells were lysed and the luciferase activity and protein concentration determined as described in Section 2.4.1

2.5   Western Blotting

Vk2/E6E7 were plated at $2.5x10^5$ cells per well in 6-well plates. Forty-eight hours after plating, cells were washed three times with PBS and then lysed using 100 μl 5X Laemmli buffer (Addendum D) per well. COS-1 cells were plated in 12-well plates at $1x10^5$ cells per well and transfected with 250 ng of the relevant receptor expression plasmid (used as positive control) using FuGENE6 transfection reagent according to the manufacturer's instructions. For the negative control, COS-1 cells were plated as above and incubated for 48 hours. Transfected and

untransfected cells were washed three times with PBS and lysed with 50-80 µl 5X Laemmli buffer (Addendum D) per well, depending on the confluency of the cells. Proteins in the lysates were denatured by boiling for 10 minutes at 95ºC prior to separation on an 8% reducing SDS-PAGE gel. A Fermentas PageRuler™ prestained protein ladder (Inqaba Biotec, Cape Town, South Africa) was used as a marker for protein size. Following electrophoresis, the proteins were electro-blotted and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Randburg, South Africa). The membranes were subsequently blocked in 5% (w/v) fat free milk powder in Tris buffered saline (TBS) containing 0.1% (v/v) Tween (TBST) (Addendum D) for 1 hour at room temperature. Membranes were subsequently incubated for 16 hours at 4ºC in the appropriate primary antibody diluted in 5% (w/v) fat free milk powder in TBST. Membranes were washed once in TBST for 15 minutes, followed by three 5 minute washes. Following the washing steps, the membranes were incubated for 1 hour and 30 minutes with the appropriate secondary antibody diluted in 5% fat free milk powder in TBST. Membranes were then washed as mentioned above. Proteins were visualized using autoradiography with Pierce® ECL Western blotting substrate (Thermo Fisher Scientific, Johannesburg, South Africa) and X-Ray film (African X-Ray Industrial & Medical (PTY) Ltd., Cape Town, South Africa). A list of antibodies used in this study and their optimal dilutions can be found in Addendum B1.

2.6   Isolation of total RNA

Vk2/E6E7 cells were seeded in 12-well plates at 1×10^5 cells per well. For studies investigating the effect of the HIV-1 accessory protein Vpr, cells were transfected with 1 µg pCMV4-3HA·Vpr using FuGENE6 transfection reagent as instructed by the manufacturers. Twenty-four hours later, cells were incubated for two hours with 0.1% (v/v) EtOH or 1 µM test compound, in the absence or presence of 0.02 µg/ml TNF-α. The cells were subsequently lysed using 400 µl TRI® Reagent

(Sigma-Aldrich Pty Ltd., Kempton Park, South Africa). The lysates were transferred to 1.5 ml microcentrifuge tubes and 80 μl chloroform was added. The solution was vortexed for 15 seconds and incubated for 2-3 minutes at room temperature. Samples were then centrifuged at 20800 x g for 15 minutes at 4ºC, thereby separating the sample into three phases: protein at the bottom, DNA in the middle and a clear aqueous phase containing the RNA on top. The aqueous phase containing the RNA was transferred to a 1.5 ml microcentrifuge tube, and an equal volume of ice-cold isopropanol was added. Samples were vortexed and incubated at room temperature for 15 minutes. The samples were then centrifuged at 20800 x g for 10 minutes at 4ºC and the supernatant was discarded. The RNA pellet was washed with 500 μl 75% (v/v) EtOH in diethyl pyrocarbonate (DEPC) treated water. The samples were vortexed and centrifuged at 6800 x g for 5 minutes at 4ºC. The supernatant was discarded and the RNA pellet air dried for 5 minutes on ice. The pellet was dissolved in 15 μl DEPC treated water. RNA was stored at -80ºC. RNA concentrations were determined using a NanoDrop 1000 (Thermo Fisher Scientific, Johannesburg, South Africa). The integrity of the RNA was determined on a denaturing formaldehyde 1% (w/v) agarose gel by confirming the presence of intact 18S and 28S ribosomal bands. A representative gel is shown in Addendum C, Figure C1.

2.7   cDNA synthesis

cDNA synthesis was performed using the transcriptor first strand cDNA synthesis kit (Roche Diagnostics Pty. Ltd., Randburg, South Africa) by following the manufacturer's guidelines. Briefly, 1 μg total RNA was added to 1 μl anchored-oligo(dT)$_{18}$ primer (final concentration of 2.5 μM) and DEPC treated water was added to a volume of 13 μl. The RNA was denatured for 10 minutes at 65ºC and then incubated on ice. The remaining components of the reverse transcription mix were added in the following order: 4 μl of 5X transcriptor reverse transcriptase reaction buffer, 0.5 μl of protector RNase inhibitor (40 U/μl), 2 μl of deoxynucleotide mix (final concentration of 1 mM per

nucleotide) and 0.5 μl of transcriptor reverse transcriptase (20 U/μl). For reverse transcription to take place the samples were incubated for 1 hour at 50ºC and the activity of the reverse transcriptase was inhibited by incubating the samples at 85ºC for 5 minutes. The cDNA samples were stored at -20ºC.

2.8   Small interfering RNA (siRNA) transfection

Vk2/E6E7 cells were seeded in 12-well plates at 1×10^5 cells per well. The next day, cells were transfected with 10 nM validated GR5 siRNA directed against the human GRα (Qiagen, Germanstown, United States of America) or a validated nonsilencing negative control (NSC) (a scrambled sequence) (Qiagen, Germanstown, United States of America) using HiPerfect transfection reagent (Qiagen, Germanstown, United States of America) as per the manufacturer's instructions. Briefly, 2.95 μl HiPerfect transfection reagent was added to 46.2 μl pre-warmed Optimem medium containing GlutaMAX™ (GibcoBRL, Paisley, Scotland) and used to dilute the siRNA to a final concentration of 10 nM. The transfection mix was incubated for 10 minutes at room temperature and added to each well in a dropwise manner. For studies investigating the effect of Vpr, cells were first transfected with GR5 and NSC as described above, and then twenty-four hours later the cells were transfected with 1 μg of pCMV4-3HA·Vpr using FuGENE6 transfection reagent according to the   manufacturer's instructions. Twenty-four hours later, all cells were incubated for two hours with either 0.1% (v/v) EtOH or 1 μM test compound, in the absence or presence of 0.02 μg/ml TNF-α. Total RNA was isolated and cDNA synthesized as described in Sections 2.6 and 2.7. The expression of the genes of interest were analysed using QPCR as described in Section 2.10. To verify protein knockdown, cells which were transfected in parallel were analysed by Western blotting, as described in Section 2.5.

2.9   Conventional polymerase chain reaction (PCR)

Conventional PCR was performed to confirm that the primers amplified a single product of the correct size. The PCR was performed on a Bio-Rad MyCycler™ Thermal Cycler (Bio-Rad Laboratories Ltd., Johannesburg, South Africa) using the GoTaq® Flexi DNA Polymerase (Promega, Madison, United States of America). The primer sequence for MIP-1α and MIP-1β amplification were obtained from Hara *et al.* (120). The specific primers used were as follows: for MIP-1α, 5'-TGCAACCAGTTCTCTGCATC-3' (forward) and 5'-TTTCTGGACCCACTCCTCAC-3' (reverse); for MIP-1β, 5'-AAGCTCTGCGTGACTGTCCT-3' (forward) and 5'-GCTTGCTTCTTTTGGTTTGG-3' (reverse). The PCR reaction mix used to amplify both MIP-1α and MIP-1β is described in Table 2.1. The thermal cycling parameters for both genes were as follows: 95ºC for 1 minute, followed by 35 cycles of denaturing at 94ºC for 30 seconds, annealing at 59ºC for 30 seconds, elongation at 72ºC for 30 seconds, and a subsequent elongation step of 72ºC for 8 minutes. PCR samples were visualised on a 2.5% agarose gel stained with ethidium bromide. A representative agarose gel showing PCR amplicons of the correct size can be found in Addendum B2, Figure B2.1.

Table 2.1: Reaction mix for conventional PCR

Reaction mix for conventional PCR	μl
Forward Primer (5 mM)	2
Reverse Primer (5 mM)	2
dNTP Mix (10 mM)	1
$MgCl_2$ (25 mM)	2
5X Green GoTaq® Flexi Buffer	5
Taq DNA polymerase (5 U/μl)	0.5
Template	1
Made up with PCR grade H_2O to final volume	25
Mineral oil	20

2.10 Quantitative realtime PCR (QPCR)

QPCR was performed using the Light-Cycler, Rapid Thermal Cycler System (Roche Diagnostics Pty. Ltd., Randburg, South Africa) and the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics Pty. Ltd., Randburg, South Africa) as specified in Addendum C Table C1. The QPCR conditions for both MIP-1α and MIP-1β were as follows: 95ºC for 15 minutes, followed by 40 cycles of denaturation at 95ºC for 10 seconds, annealing at 59ºC for 20 seconds, and elongation at 72ºC for 30 seconds. The primer sequences for MIP-1α and MIP-1β are described in Section 2.9 Since the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is not influenced by the test compounds, it was used as an internal control. The primer set used for GAPDH is as follows: 5'-TGAACGGGAAGCTCACTGG-3' (forward) and 5'-TCCACCACCCTGTT-GCTGTA -3' (reverse) (121), and the QPCR conditions were: 95ºC for 10 minutes, followed by 24 cycles of denaturation at 95ºC for 10 seconds, annealing at 59ºC for 10 seconds, and elongation at 72ºC for 12 seconds. The melting curve analysis was performed to confirm the amplification of a single product in each sample (Addendum C, Figure C3). The amplification efficiency for each primer set as well as the relative transcript levels were determined as described by Pfaffl *et al.* (122). The primer efficiencies were 2.52, 2.54 and 1.84 for MIP-1α, MIP-1β and GAPDH, respectively. A detailed discussion of the QPCR method can be found in Addendum C.

2.11 Whole cell binding assay

Competitive whole cell binding assays were performed essentially as described by Bamberger *et al.* (123) with the following modifications. Vk2/E6E7 cells were plated at $2x10^5$ cells/well in a 12-well

plate (AEC-Amersham, Cape Town, South Africa). On day 3, the cells were washed with PBS and incubated for 2 hours at 37ºC with 10 nM [^{3}H]-E$_2$ (110 Ci/mmol) in the absence (total binding) or presence of 10 μM unlabelled E$_2$ (non-specific binding). The cells were placed on ice and washed 3 times for 15 minutes with ice-cold PBS containing 0.2% (w/v) bovine serum albumin (BSA). Cells were then lysed with 100 μl passive lysis buffer (Promega, Madison, United States of America). Lysates were added to 1.5 ml Flo-Scint II Scintillation fluid (PerkinElmer Life and Analytical Science, South Africa) and the total binding determined by scintillation counting as counts per minute (cpm) determined using a Packard 1900CA Tri-Carb liquid scintillation counter with a counting efficiency of 40%. Specific binding was determined by subtracting the non-specific binding from the total binding. Protein concentrations were determined using the Bradford assay method (119). Specific binding was normalised to protein concentration. The reported values are averages of at least three independent experiments, with each condition performed in triplicate. The receptor number in femtomole per milligram (ƒmol/mg) protein was determined as follows:

The specific activity of [^{3}H]-E$_2$ (110 Ci/mmol) was converted to disintegrations per minute (dpm) per mmole, by multiplying by a factor of 2.22×10^{12} (1 Ci equals 2.22×10^{12} dpm). The dpm was multiplied by the counting efficiency to get the cpm per mmole, and divided by 10^{12} to get the cpm/ƒmol. The counting efficiency was 40% in our system. The specific activity was expressed as cpm/ƒmol. Dividing the specific binding cpm value by this value gives specific binding in ƒmol, which was divided by the protein content of the sample in mg, to give the number of binding sites in ƒmol/mg protein.

CHAPTER 3 : RESULTS

3.1   MPA and Net-A differentially regulate the expression of MIP-1α and MIP-1β

The human vaginal cell line, Vk2/E6E7, was treated with 0.02 μg/ml TNF-α for varying times and the expression of MIP-1α and MIP-1β mRNA determined by conventional PCR (Figure 3.1). TNF-α, a pro-inflammatory cytokine, has previously been shown to increase the expression of MIP-1α and MIP-1β in human fetal microglia as well as rat epithelial cells (124,125). Similarly, Fichorova *et al.* showed that TNF-α induces MIP-1β protein secretion in the Vk2/E6E7 cell line (56). The results in Figure 3.1 shows that the optimal TNF-α-induced expression of MIP-1α is at 2 hours. Similar results were observed for MIP-1β (results not shown). However, the conventional PCR results could not be quantified as the expression of an internal control, such as GAPDH, was not determined. For this reason, the experiment was repeated using QPCR. In agreement with the conventional PCR results, QPCR showed optimal TNF-α-induced expression of MIP-1α (Figure 3.2) and MIP-1β (data not shown), at 2 hours. Cells were thus incubated for 2 hours in all subsequent experiments.

The Vk2/E6E7 cells were subsequently treated with 0.02 μg/ml TNF-α in the absence or presence of P4, MPA and Net-A for 2 hours, followed by QPCR analysis for the expression of MIP-1α and MIP-1β (Fig 3.3A). Although not significant, MPA seems to upregulate TNF-α-induced MIP-1α expression (1.3-fold). In contrast, P4 significantly (p<0.01) increased MIP-1α expression (2.2-fold), while Net-A had no effect (Figure 3.3A). Conversely, both P4 and MPA significantly increased the expression of MIP-1β (p<0.05 and p<0.01, respectively) as shown in Figure 3.3B. In agreement with the effect of Net-A on the expression of MIP-1α mRNA, Net-A had no effect on MIP-1β gene

expression (Figure 3.3B). Taken together, MPA and Net-A showed differential regulation on the expression of MIP-1α and MIP-1β.
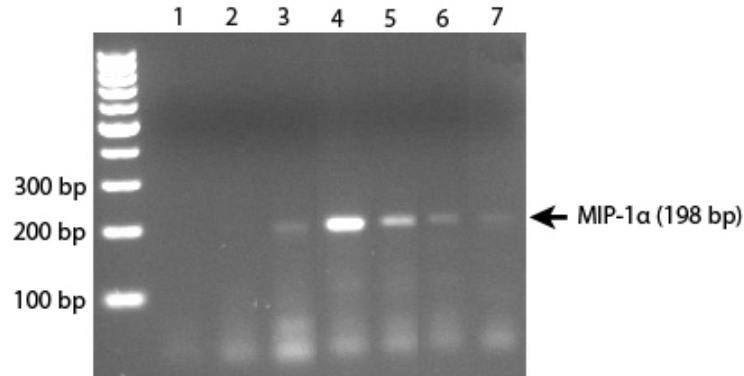

Figure 3.1: Time course showing optimal induction of TNF-α-induced expression of MIP-1α. Vk2/E6E7 cells were treated with 0.1% EtOH in the absence or presence of 0.02 μg/ml TNF-α for varying times. Total RNA was isolated, cDNA synthesized, and analysed using conventional PCR with primers specific to MIP-1α. Products were electrophoresed on a 2% agarose gel and visualised using etidium bromide staining. Lane 1 represents the no template control and lane 2, a sample induced with 0.1% EtOH. Lanes 3-7 represents the PCR amplicons after treatment with 0.1% EtOH in the presence of 0.02 μg/ml TNF-α for 30min, 2h, 4h, 8h and 24h, respectively.
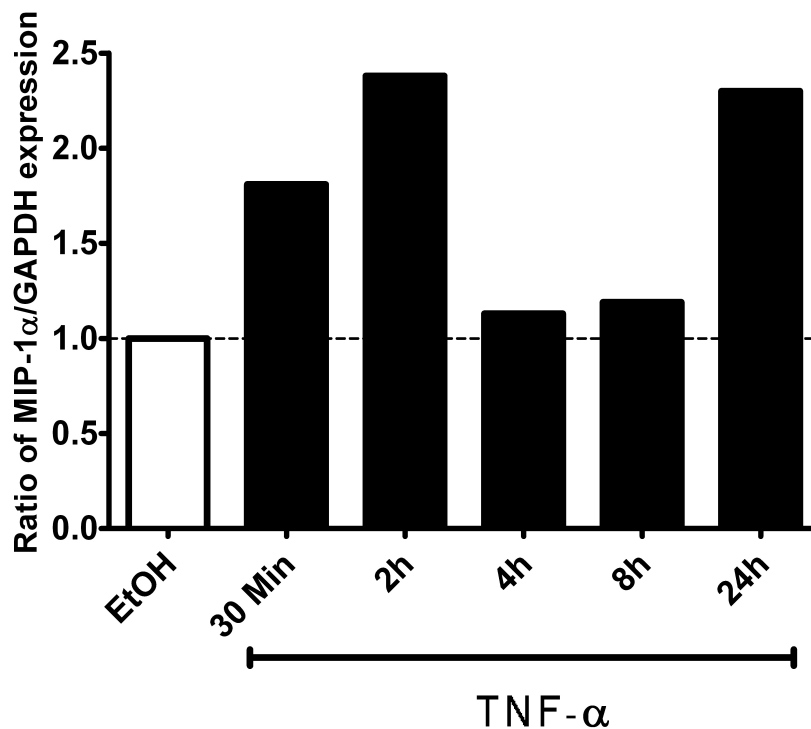
Figure 3.2: Optimal TNF-α-induced expression of MIP-1α at 2 hours. Vk2/E6E7 cells were treated with 0.1% EtOH in the absence or presence of 0.02 μg/ml TNF-α for varying times. Total RNA was isolated, cDNA synthesized, and analysed using QPCR with primers specific to MIP-1α and the internal control, GAPDH. Relative expression of MIP-1α was normalised to relative GAPDH gene expression. The result represents a single experiment.

A)
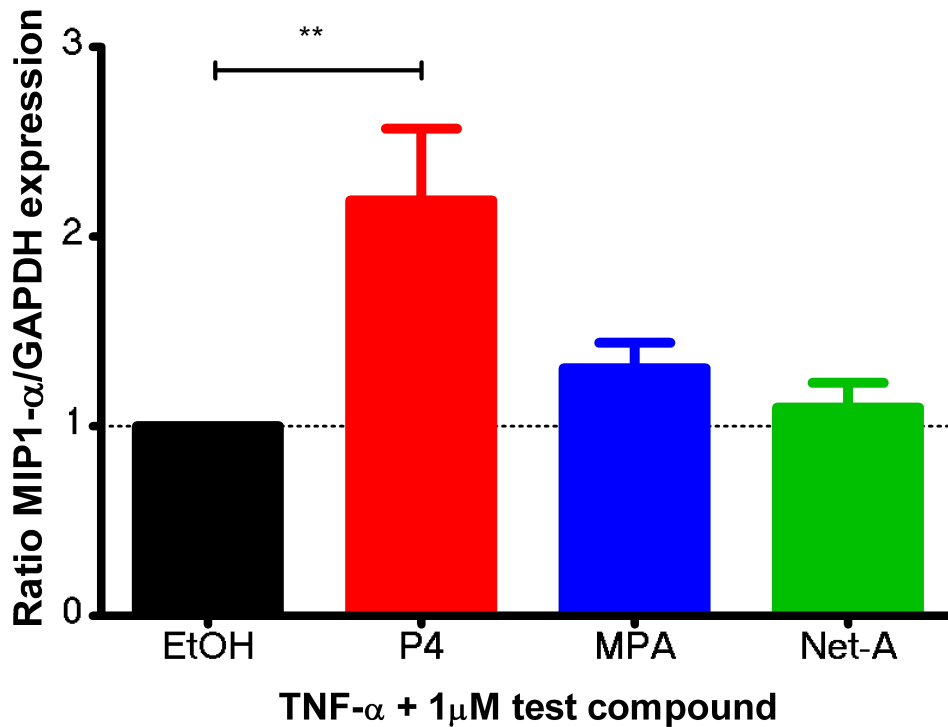
B)
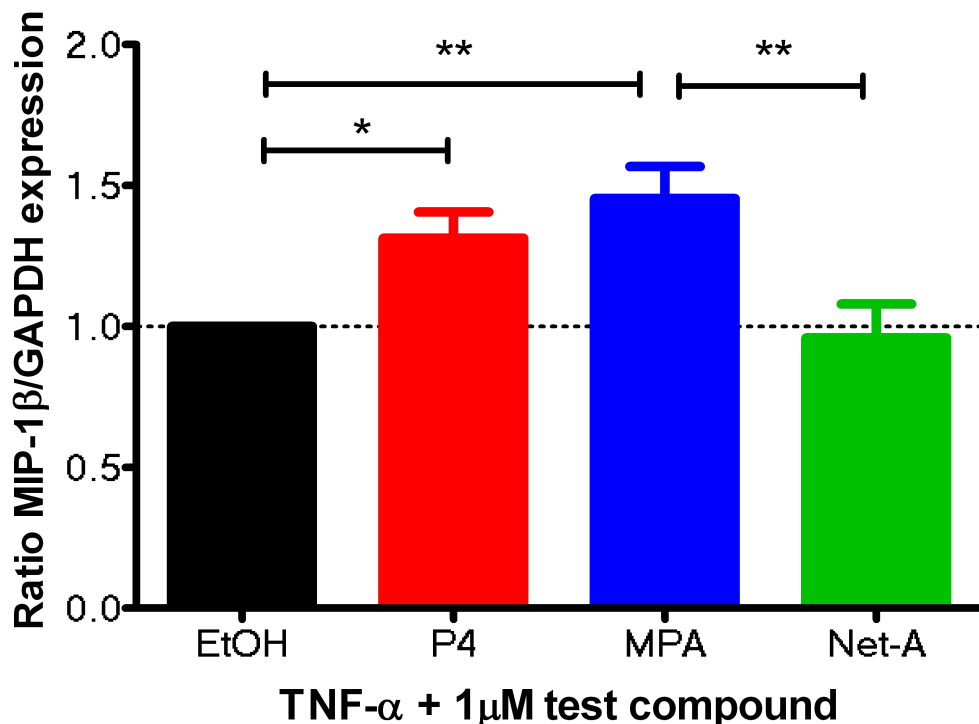
Figure 3.3: The effects of P4, MPA and Net-A on the TNF-α-induced mRNA expression of MIP-1α and MIP-1β in the human Vk2/E6E7 cell line. Vk2/E6E7 cells were incubated with 0.02 μg/ml TNF-α in the presence of 0.1% EtOH or 1 μM P4, MPA or Net-A for 2 h. Total RNA was isolated, cDNA synthesized, and analysed using QPCR with primers specific to **(A)** MIP-1α, **(B)** MIP-1β, and the internal control, GAPDH. Relative expression of MIP-1α and MIP-1β was normalised to the relative GAPDH expression. Statistical analysis was performed using one-way ANOVA with Bonferroni Multiple Comparison post tests (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$). Results shown are the averages (±SEM) of at least six independent experiments.

3.2   Vk2/E6E7 cells express both ER-α and ER-β

As P4 and MPA are known to interact with the PR, GR, AR and MR (31,34,67,81,82), any of these receptors could be involved in the regulation of MIP-1α and MIP-1β mRNA expression by P4 and MPA. It has previously been shown that the PR, GR, AR and low levels of MR, are expressed in the Vk2/E6E7 cells (13).

However, whether this cell line also expresses the ER, another member of the steroid receptor family, had not been determined in the above-mentioned study (13). This is relevant as whether MPA interacts with the ER is controversial (126–129). The presence of the ER in this cell line was thus investigated using a combination of whole cell binding (Table 3.1) and Western blotting (Figure 3.4). The whole cell binding results with tritiated E_2 show that endogenous ER is expressed in the Vk2/E6E7 cell line. The number of ER binding sites, in ƒmol/mg protein, was calculated from the specific activity of ^3H-E_2 and the specific binding cpm value, as described in Section 2.11. The levels of ER (7.12 ƒmol/mg protein) in Vk2/E6E7 cells are similar to those of the AR and GR [(11); reproduced in Addendum F]. There are two functional isoforms of the ER, namely ERα and ERβ (130). Western blot analysis using antibodies specific for ERα and ERβ, respectively, indicated the presence of both isoforms in the Vk2/E6E7 cell line (Figure 3.4). I performed a number of Western blot analyses to show the expression of the ERα isoform, but was unable to obtain a clear blot. Thus, the Western blot showing the expression of the ERα isoform in this study is blurred. However, Africander *et al.* [(11), Addendum F] previously clearly showed the expression of the ERα isoform in the Vk2/E6E7 cell line. Taken together, the PR, GR, AR, ER and possibly MR, are expressed in the human Vk2/E6E7 cell line [(11); Addendum F].

Table 3.1: ER expression levels in Vk2/E6E7 cells[1]

TB (%)	NSB (%)	SB (%)	Level of ER ($f$mol/mg protein)
100	72.26	28.04	7.12

[1]Vk2/E6E7 cells were incubated with 10 nM ^{3}[H]-E_2 in the absence (total binding, TB) and presence (non-specific binding, NSB) of unlabelled E_2. Specific binding (SB) [TB-NSB] was normalised to the protein concentration. The data was obtained from three independent experiments with each condition performed in triplicate.

3.3   Expression and functional activity of endogenous GR in the Vk2/E6E7 cells

As it is known that the Vk2/E6E7 cell line expresses the PR, GR, AR, and ER, the strategy was to determine which of these steroid receptors mediate the effect of MPA on the expression of the MIP-1α and MIP-1β genes. Since MPA has previously been shown to be a partial agonist for transactivation via the GR (31,34), while Net-A displays no GR agonist activity, it was hypothesised that the effect of MPA on MIP-1α and MIP-1β gene expression is mediated by the GR. To be able to test this hypothesis, the reported expression of a functional GR in the Vk2/E6E7 cell line (13) was first investigated. Using Western blot analysis, with a GRα specific antibody, the expression of endogenous GR was detected (Figure 3.5A).
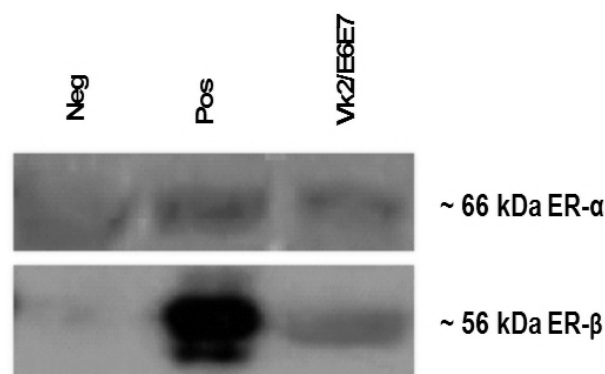
Figure 3.4: The ER-α and ER-β isoforms are expressed in the human Vk2/E6E7 cell line. Whole cell extracts of the Vk2/E6E7 cell line (Vk2/E6E7) were analysed on separate blots by Western blotting with ERα and ERβ specific antibodies, respectively. Whole cell extracts of untransfected COS-1 cells were used as a negative control (Neg). Positive controls (Pos) are whole cell extracts of COS-1 cells transiently transfected with the pSG5ERα or pSG5ERβ expression vectors, respectively.

A)
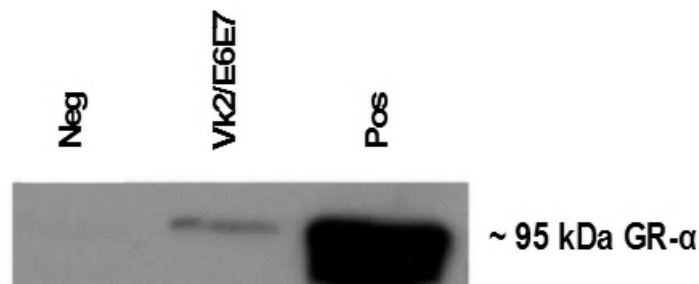
B)
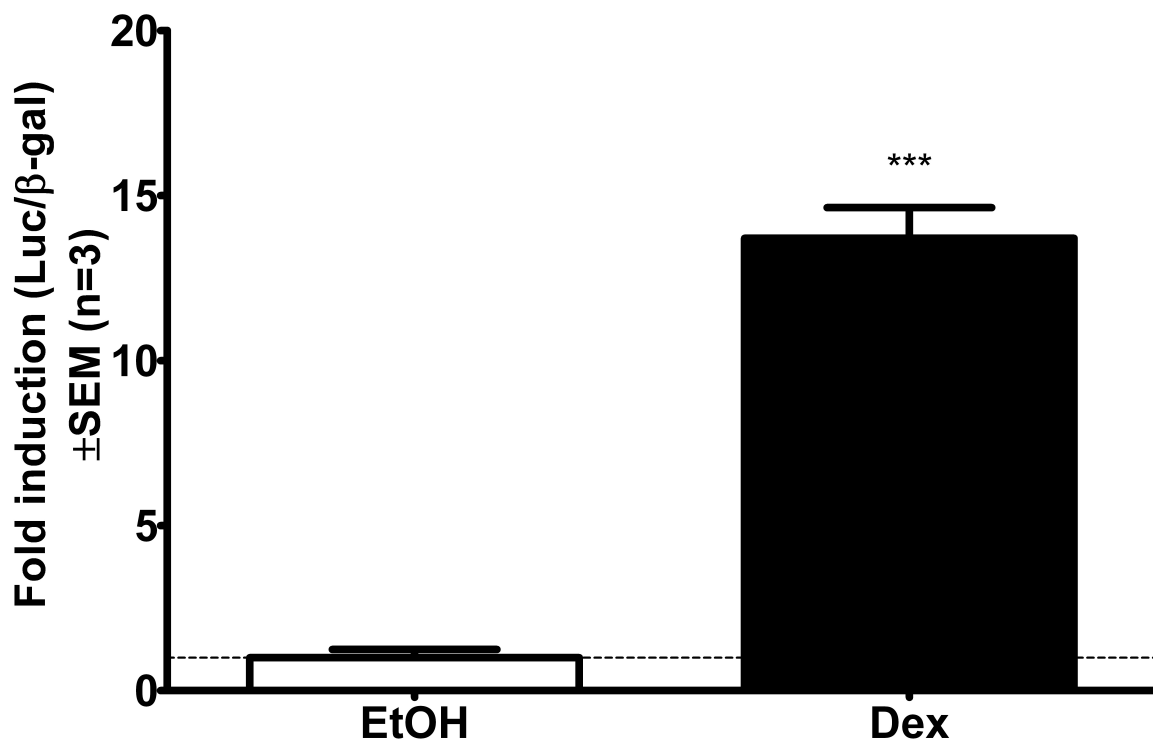
Figure 3.5: Expression of a functional GRα in the human Vk2/E6E7 cell line. (A) Western blot showing expression of GRα in the Vk2/E6E7 cell line. Whole cell extracts of the Vk2/E6E7 cell line (Vk2/E6E7) were analysed by Western blotting with a GRα specific antibody. Whole cell extracts of untransfected COS-1 cells were used as a negative control (Neg), while whole cell extracts of COS-1 cells transfected with the pRS-hGRα expression vector was used as a positive controls (Pos). **(B) Transactivation of a transiently transfected GRE-containing promoter-reporter construct via endogenous GR.** Vk2/E6E7 cells seeded at $5x10^4$ per well in a 24-well plate were transfected with 300 ng pTAT-GRE2-Elb-Luc, 30 ng pSV-βgal and 300 ng pGL2Basic (empty vector control) using Fugene6 transfection reagent according to the manufacturer's instructions. Twenty-four hours after transfection, cells were treated with either 0.1% EtOH or 10 μM Dex for 24h. The result is the average (±SEM) of three independent experiments with each condition performed in triplicate. Statistical analysis was performed using the unpaired two tailed t-test (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

Next a promoter-reporter assay was used to investigate whether the endogenous GR expression in

the Vk2/E6E7 cell line, is transcriptionally active. The cells were transiently transfected with a

GRE-driven reporter construct containing two copies of the rat TAT-GRE (pTAT-GRE2-Elb-luc) and subsequently treated with 10 μM of the GR-specific agonist, Dex.

The result shows a 14-fold induction in the presence of Dex, indicating that the endogenous GR in the Vk2/E6E7 cell line is transcriptionally active (Figure 3.5B).

Taken together, these results are in agreement with those of Africander (13), showing the expression of a functional endogenous GR in the Vk2/E6E7 cell line.

3.4 The GR plays a role in the regulation of MIP-1α, but not MIP-1β, by P4 and MPA in the Vk2/E6E7 cell line

Having confirmed that a functional GR is expressed in the Vk2/E6E7 cell line, it was investigated whether the GR plays a role in mediating the effects of MPA, and possibly P4, on MIP-1α and MIP-β gene expression. The GR protein expression was reduced by transfecting the Vk2/E6E7 cells with a GR specific siRNA sequence. As a control, cells were transfected with nonsilencing control (NSC) oligonucleotides. A representative Western blot verified that the GR was successfully knocked down (Figure 3.6A) by approximately 40% in the Vk2/E6E7 cells transfected with the GR5 siRNA (Figure 3.6B). When comparing cells transfected with the NSC siRNA sequence with untransfected cells (UT), the results indicate that the endogenous GR levels were not altered by transfection with NSC oligonucleotides (Figure 3.6A).

The results of GR siRNA experiment were normalised to the vehicle control of each transfection condition (NSC vs GR siRNA) as shown in Figure 3.7. These results show that, although not statistically significant, the knockdown of the GR diminished the P4-induced expression of MIP-1α,

indicating a partial role for the GR in P4-mediated induction of MIP-1α mRNA expression. A possible explanation for the lack of statistical significance for the P4 effect on MIP-1α, is the huge variation in the effect of Dex. The Bonferroni correction, used in the statistical analysis of this result, does not account for the independence of the tests, thus the probability of one test being non significant will increase the probability of another test thus displaying no significant difference. Thus, the huge error shown for the effect of Dex on the expression of MIP-1α, may explain why the difference shown by P4, is not significant. The reduction in the GR protein levels appeared to abrogate MPA-induced MIP-1α mRNA expression, suggesting a role for the GR in MPA-mediated induction of MIP-1α gene expression. Since Net-A had no effect on MIP-1α expression, it was not surprising that the decrease in GR expression had no effect on the expression of MIP-1α in the presence of Net-A.

Although not significant, the repression of MIP-1α gene expression by the GR specific agonist, Dex, was abolished by GR knockdown (Figure 3.7A). Surprisingly, Figure 3.7B shows that reducing the GR levels did not alter the effect of either P4 or MPA on MIP-1β gene expression. However, the effect of Dex, which served as a positive control, was inhibited by the decrease in GR protein levels. Interestingly, although Net-A had no effect on MIP-1β mRNA expression, a decrease in GR protein levels caused an increase in MIP-1β mRNA expression in the presence of Net-A. In summary, although not statistically significant, the results suggest that the GR is required for the upregulation of MIP-1α gene expression by P4 and MPA, but not for the upregulation of MIP-1β gene expression.

3.5   The HIV-1 accessory protein, Vpr, modulates P4- and MPA-induced expression of MIP-1α and MIP-1β

Having established that the GR most likely plays a role in P4- and MPA-induced expression of MIP-1α in the human vaginal epithelial cell line, and considering the fact that both the GR and MPA have previously been implicated in playing a role in HIV-1 pathogenesis (39,44,94,131), it was next investigated whether the HIV-1 accessory protein, Vpr, could modulate the GR-mediated transcription of MIP-1α by P4 and MPA. Although the GR did not mediate the effects of P4 and MPA on MIP-1β, the influence of Vpr on this gene was also investigated (results in Addendum E, Figure E2) in the light of previous reports showing a link between MIP-1β and Vpr in human primary lymphocytes and macrophages (117,118).

Vk2/E6E7 cells were transfected with the pCMV4-3HA·Vpr expression vector and incubated with 0.02 µg/ml TNF-α in the presence of 0.1% (v/v) EtOH or 1µM test compound. Results show that Vpr abolishes the effects of P4, and possibly MPA and Dex on MIP-1α mRNA expression (Figure 3.8). However, statistical analysis indicate that only the Vpr modulation of P4-induced MIP-1α gene expression is statistically significant (p<0.001). In contrast, Vpr did not modulate MIP-1α gene expression in the presence of Net-A. Taken together, the results suggest that the HIV-1 accessory protein, Vpr, modulates the response of P4, and possibly MPA, on MIP-1α gene expression.

A)
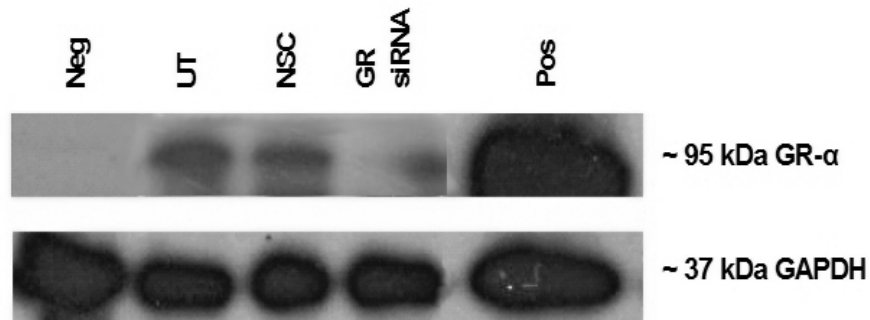
B)
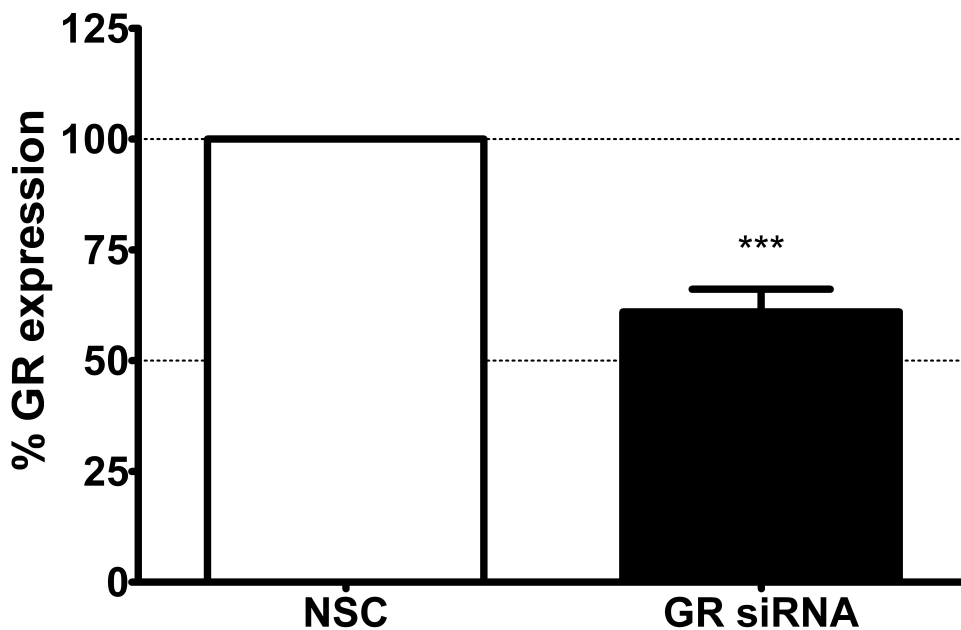
Figure 3.6: Reduction of the endogenous GR protein in the Vk2/E6E7 cell line. (A). For verification of GR knockdown, Vk2/E6E7 cells were transfected with either 10 nM nonsilencing control (NSC) or GR specific siRNA (GR siRNA), followed by preparation of whole cell extracts. Samples were analysed by Western blot using a GRα specific antibody. Untransfected COS-1 cells were used as a negative control (Neg), while COS-1 cells transfected with the pRS-hGRα expression vector were used as a positive control (Pos). Untransfected Vk2/E6E7 cells are indicated by UT. GAPDH was used as a loading control and the levels analysed by using a GAPDH specific antibody. A representative figure of four independent experiments is shown. **(B)** Western blots of four independent experiments were quantified using UN-SCAN-IT software to determine the percentage GR protein knockdown. Statistical analysis was performed using the unpaired two tailed t-test (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

A)
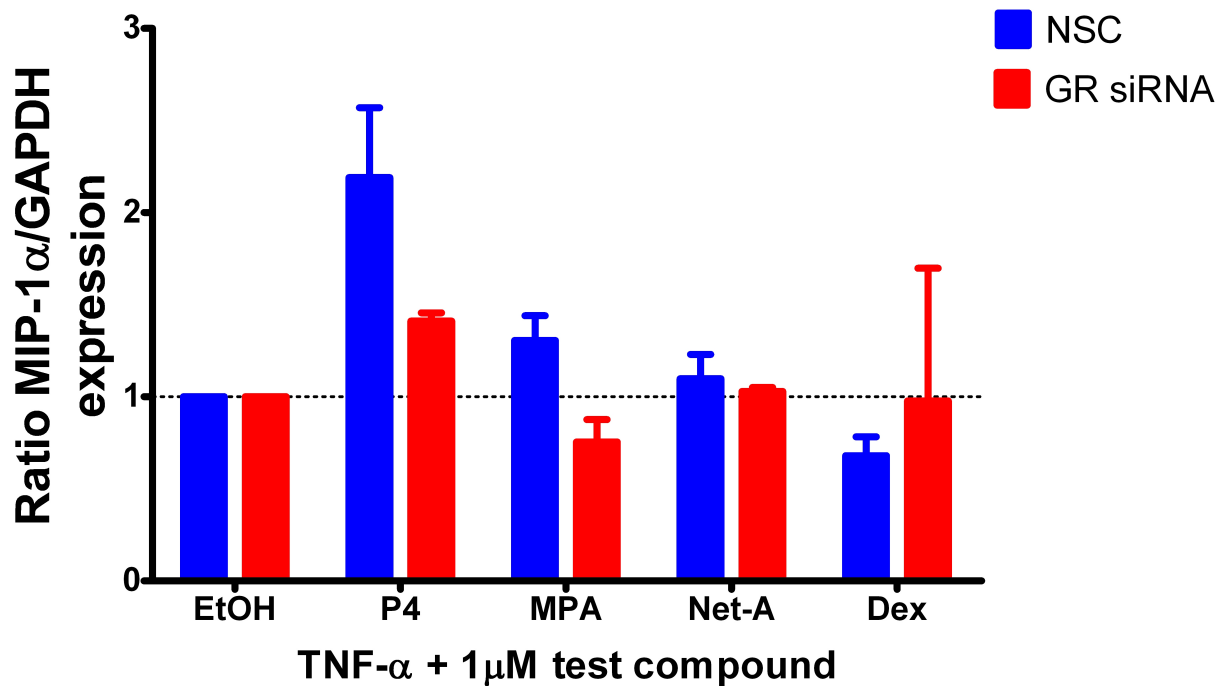
B)
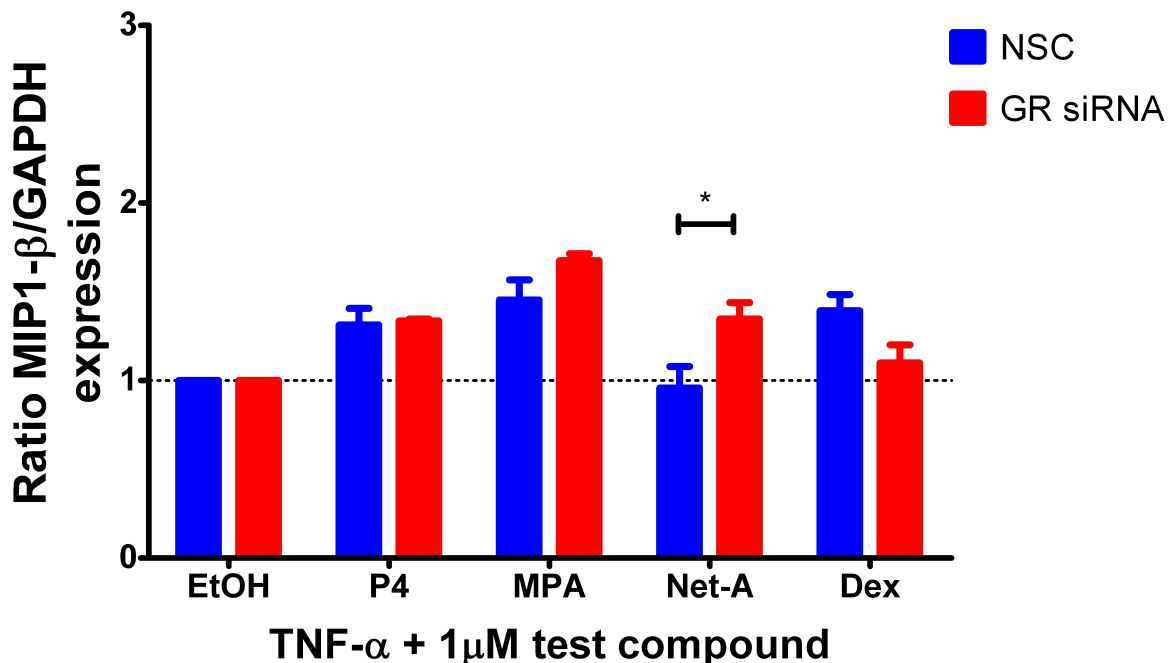
Figure 3.7: The effect of silencing GR expression by siRNA on TNF-α induced mRNA expression of MIP-1α and MIP-1β in response to P4, MPA and Net-A in the Vk2/E6E7 cell line. Vk2/E6E7 cells were transfected with 10 nM GR specific or NSC siRNA sequences. Fourty-eight hours later, cells were incubated with 0.02 μg/ml TNF-α and 1μM P4, MPA, Net-A or Dex for 2h. Total RNA was isolated, cDNA synthesized, and analysed using QPR with primers specific to **(A)** MIP-1α, **(B)** MIP-β, and the internal control, GAPDH. Relative expression of MIP-1α and MIP-1β was normalised to relative GAPDH expression. Each different transfection condition was normalised to the relevant EtOH, which was set as 1 (i.e. all the blue bars were normalized to NSC (EtOH) and all the red bars normalized to GR siRNA (EtOH)). For statistical analysis two-way ANOVA was used with Bonferroni as post test (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$). The result represents the average (±SEM) of at least two independent experiments.

3.6   The GR is involved in Vpr's influence of MPA's effect on MIP-1α

Having determined that Vpr modulates the P4- and most likely also the MPA-induced mRNA expression of MIP-1α, it was next determined whether the Vpr response is GR-dependent. Vk2/E6E7 cells were firstly transfected with the pCMV4-3HA·Vpr expression vector, and twenty-four hours later with the GR-specific or NSC siRNA sequences. Cells were then treated with 0.02 µg/ml TNF-α in the presence of 0.1 % (v/v) EtOH or 1 µM test compound. A representative Western blot verified that the GR was significantly knocked down (Figure 3.9A). The Western blots were quantified and surprisingly the result show approximately 95% reduction in the levels of endogenously expressed GR in the presence of Vpr, compared to 40% reduction in the absence of Vpr (Figure 3.9B).

The silencing of the GR appeared to modulate the effect of Vpr on P4 and MPA-induced mRNA expression of MIP-1α (Figure 3.10), suggesting that the effect of Vpr may be dependent on the liganded GR. The Dex response in the presence of Vpr also appears to be slightly altered in the absence of the GR. However, these results were from a single experiment and statistical significance could thus not be determined. Surprisingly, a large decrease in P4-induced MIP-1α mRNA expression was observed in the presence of GR siRNA, which may suggest that in the absence of GR, Vpr may interact with an alternate steroid receptor to repress MIP-1α gene expression. In summary, the results indicate that Vpr negates MPA- and possibly Dex-induced expression of MIP-1α mRNA via an interaction with the GR. However, this is the result of a single experiment and should be confirmed by further experiments before any definitive conclusions can be drawn.
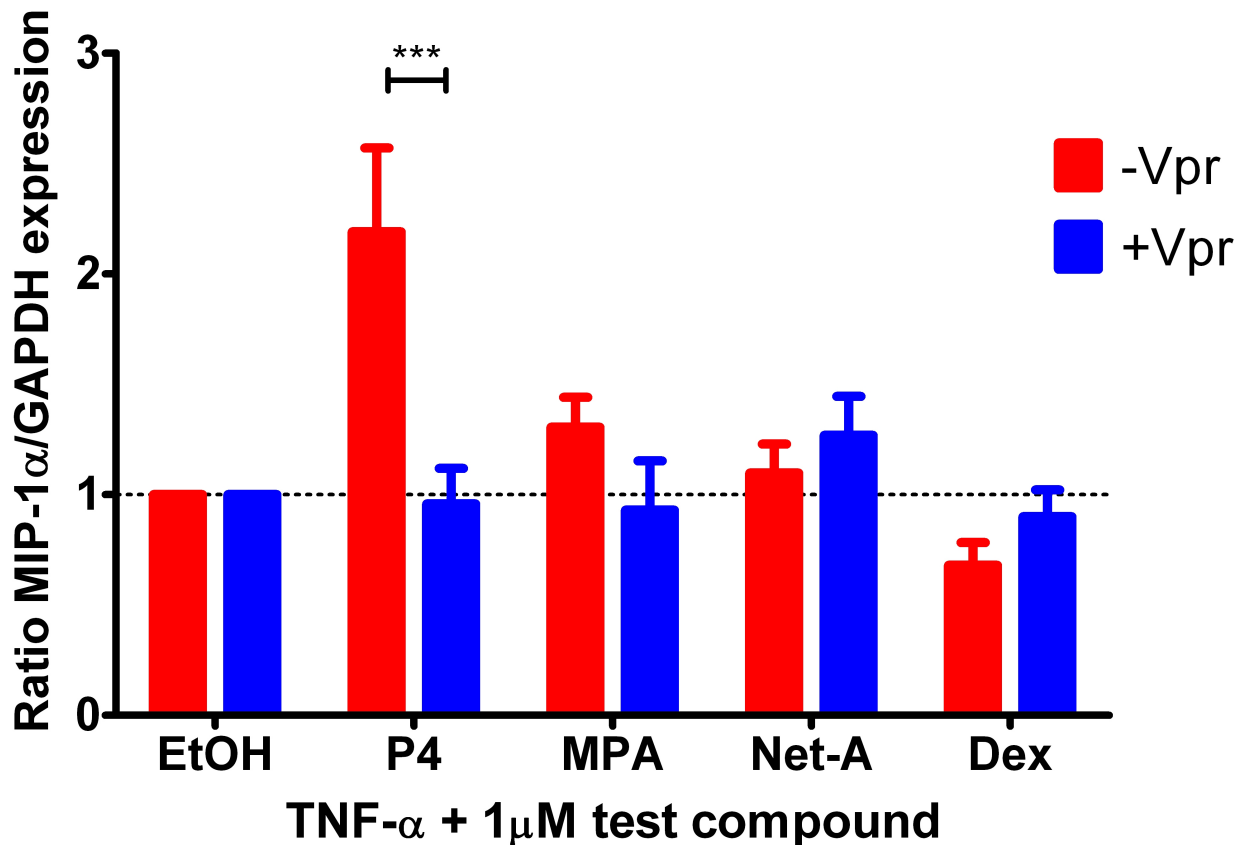
Figure 3.8: The influence of Vpr on P4, MPA and Dex induced mRNA expression of MIP-1α in the Vk2/E6E7 cell line. Vk2/E6E7 cells were transfected with 1000 ng pCMV4-3HA·Vpr. Twenty-four hours later, cells were incubated with 0.02 μg/ml TNF-α and 1μM P4, MPA, Net-A or Dex for 2h. Total RNA was isolated, cDNA synthesized, and analysed using QPCR with primers specific to MIP-1α and the internal control, GAPDH. Relative expression of MIP-1α was normalised to relative GAPDH expression. Each different transfection condition was normalised to the relevant EtOH, which was set as 1 (i.e. all the blue bars were normalized to (EtOH) -Vpr and all the red bars normalized to (EtOH) +Vpr). For statistical analysis two-way ANOVA was used with Bonferroni as post test (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$). The result represents the average (±SEM) of at least two independent experiments.

Figure 3.11 summarises the effects of P4 and MPA on MIP-1α mRNA expression using the data from Figure 3.7A, 3.8 and 3.10, and shows that the upregulation in the expression of MIP-1α by both P4 and MPA is mediated by the GR. In addition, it shows that Vpr inhibits P4- and MPA-induced expression of MIP-1α, and that the expression is repressed in the absence of GR. An interpretation of these results is proposed in a model in Chapter 4.

3.7   Vpr modulates Dex and MPA glucocorticoid agonist activity for transactivation on a reporter gene

The finding that Vpr appears to require the GR for its effects on MIP-1α gene expression in response to MPA and possibly Dex, raises the question whether Vpr could influence the agonist potency and efficacy for transactivation via the GR. To this end, a promoter reporter assay was performed in COS-1 cells, which are devoid of steroid receptors, thus making them an ideal system for investigating effects via only the GR. COS-1 cells were transiently transfected with the pRS-hGRα expression vector, the GRE-driven reporter construct pTAT-GRE2-Elb-Luc, the pCMV4-3HA·Vpr vector or pGL2-Basic, an empty vector. The cells were subsequently incubated with increasing concentrations of Dex and MPA.

In the absence of Vpr, both Dex and MPA showed dose dependent transcriptional activity of the GR, via a GRE, Figure 3.12. The presence of Vpr significantly increased the efficacies of both Dex ($p<0.01$) and MPA ($p<0.05$). Furthermore, the potency of Dex was significantly increased ($p<0.05$) in the presence of Vpr, but Vpr had no effect on the potency of MPA via the GR (Figure 3.12). The potencies and efficacies of Dex and MPA via the GR in the absence and presence of Vpr are shown in Table 3.2, and indicates that Vpr increases the efficacy of the GR ligands, Dex and MPA, while decreasing the potency of the GR specific agonist Dex, and not the partial agonist MPA.

A)
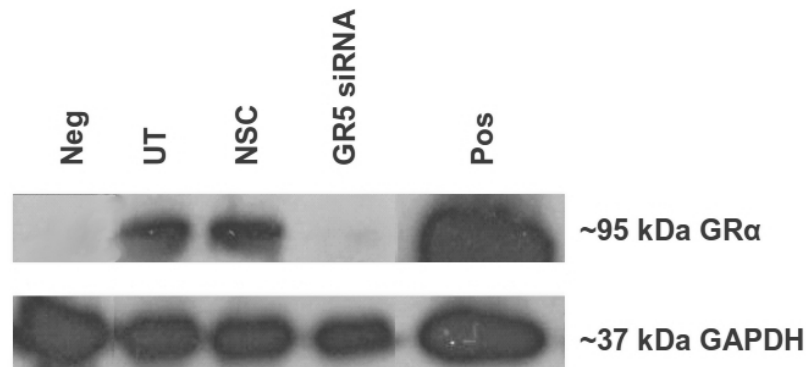
B)
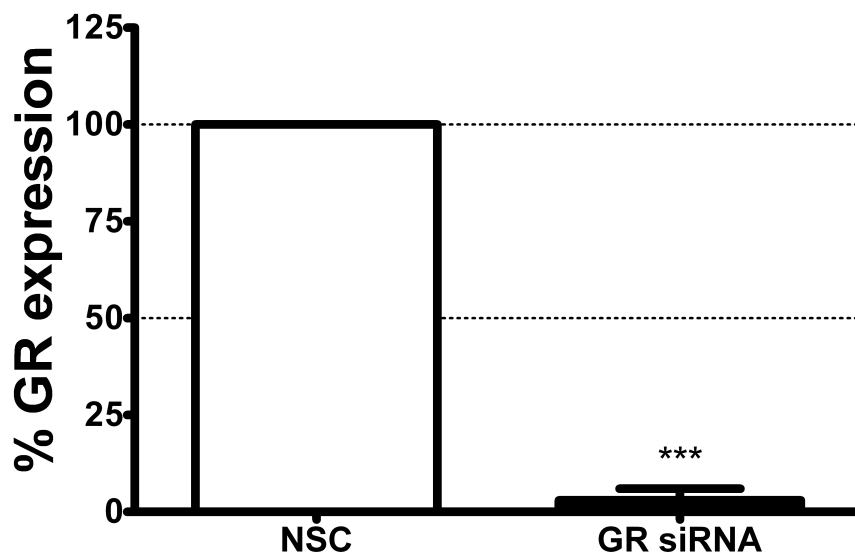
Figure 3.9: Reduction of the GR protein in the Vk2/E6E7 cell line in the presence of Vpr. For verification of GR knockdown, whole cell extracts of Vk2/E6E7 cells transfected with 1000 ng pCMV4-3HA·Vpr and 24h later with either 10 nM NSC or GR specific siRNA (GR siRNA), were analysed by Western blot using a GRα specific antibody. Untransfected COS-1 cells were used as a negative control (Neg) while COS-1 cells transfected with the pRS-hGRα expression vector were used as positive controls (Pos). Untransfected Vk2/E6E7 cells are indicated by UT. GAPDH was used as a loading control and the levels analysed by using a GAPDH specific antibody. A representative figure of four independent experiments is shown. **(B)** Western blots of three independent experiments were quantified using UN-SCAN-IT software to determine the percentage GR protein knockdown. Statistical analysis was performed using the unpaired two tailed t-test (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$).
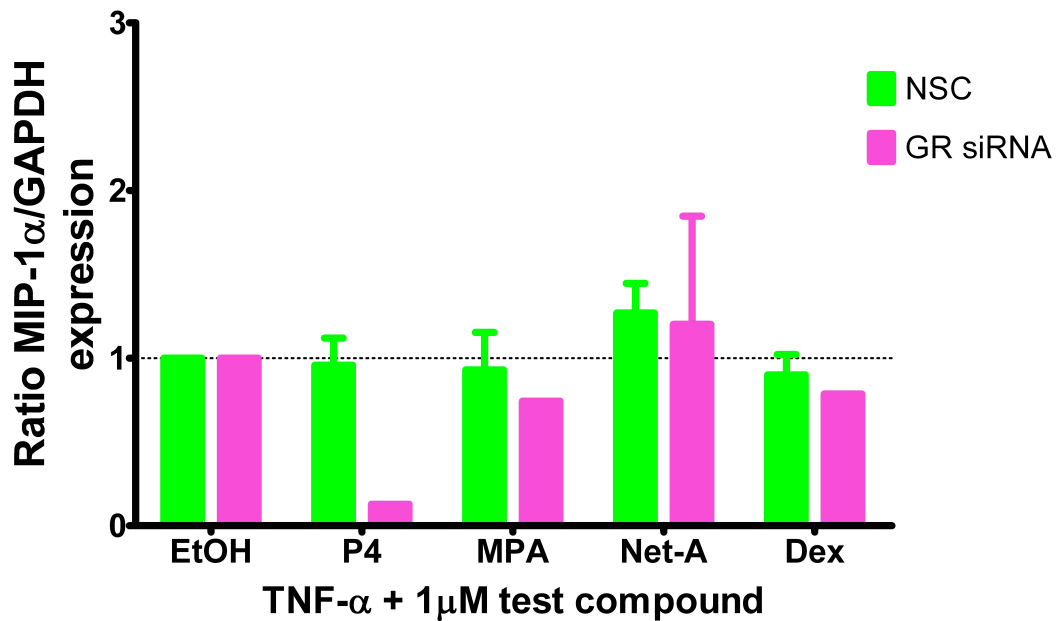
Figure 3.10: The effect of silencing GR expression by siRNA on TNF-α induced expression of MIP-1α in response to P4, MPA and Net-A in the Vk2/E6E7 cell line in the presence of Vpr. Vk2/E6E7 cells were transfected with 1000 ng pCMV4-3HA·Vpr and 24h later with 10 nM GR siRNA or NSC (a control). Forty-eight hours later, cells were incubated with 0.02 μg/ml TNF-α and 1μM P4, MPA, Net-A or Dex for 2h. Total RNA was isolated, cDNA synthesized, and analysed using QPCR with primers specific to MIP-1α and the internal control, GAPDH. Relative expression of MIP-1α was normalised to relative GAPDH gene expression. Each different transfection condition was normalised to the relevant EtOH, which was set as 1 (i.e. all the green bars were normalized to NSC (EtOH) and all the pink bars normalized to GR siRNA (EtOH)). For statistical analysis two-way ANOVA was used with Bonferroni as post test (* $p<0.05$, **
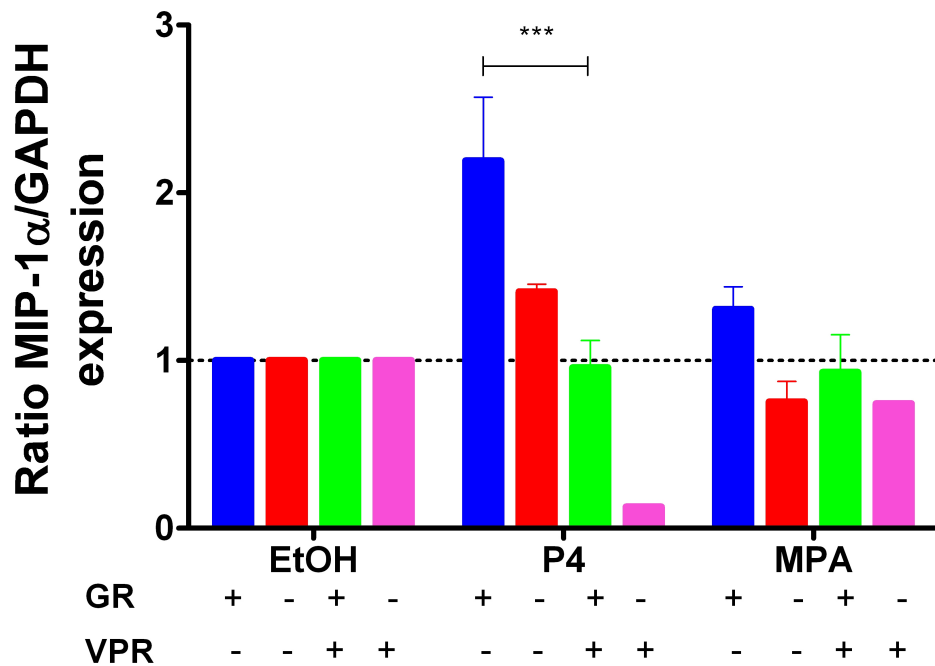
Figure 3.11: Summary of the effects of P4 and MPA on MIP-1α mRNA expression in Vk2/E6E7. The figure is a summary of P4 and MPA effects on MIP-1α expression using data from Figures 3.7A, 3.8 and 3.10. Each different transfection condition was normalised to the corresponding EtOH, which was set as 1 (i.e. the blue bars were normalized to +GR -Vpr (EtOH), the red bars normalized to -GR -Vpr (EtOH), the green bars were normalized to +GR +Vpr (EtOH), and the pink bars normalized to -GR +Vpr (EtOH)).

A)
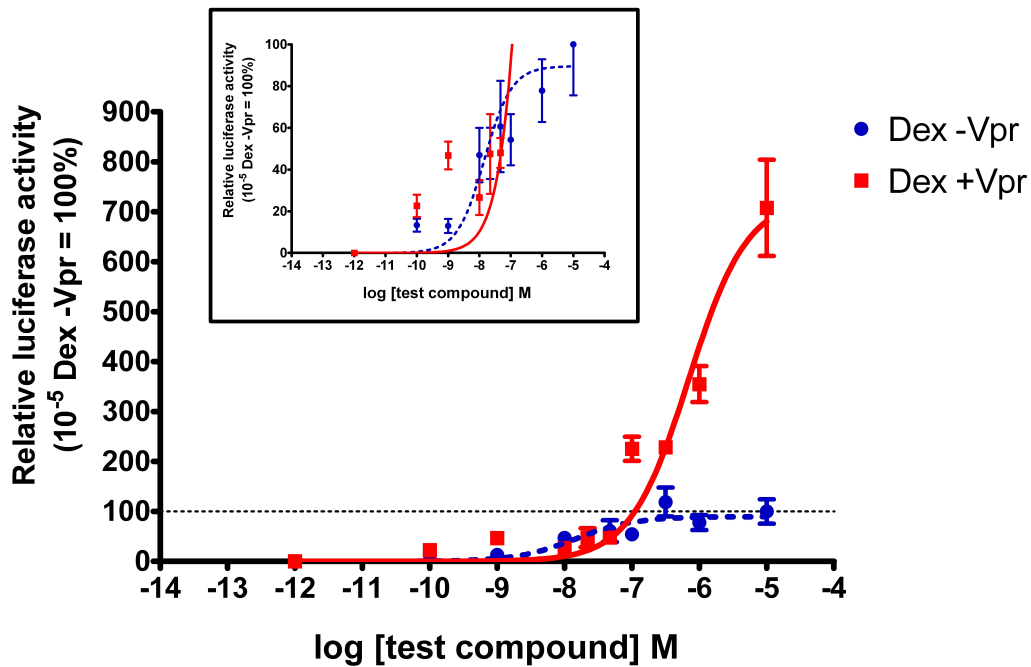
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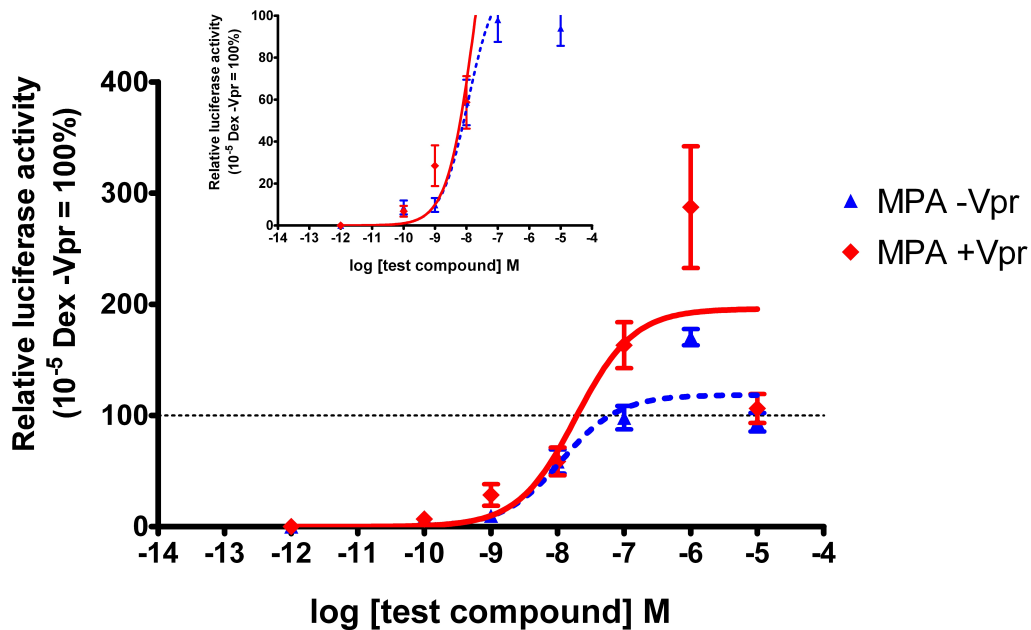
Figure 3.12: The influence of Vpr on the dose dependent effect of Dex and MPA on a synthetic GRE via the GR. COS-1 cells were transfected with 900 ng of pRS-hGRα, 9000 ng pTAT-GRE-E1b-luc plasmid, 900 ng pSV-βgal and either 900 ng pCMV4-3HA·Vpr or pGL2-Basic (empty vector control), using Fugene6 transfection reagent according to the manufacturer's instructions. Cells were incubated for 24h in the absence and presence of increasing concentrations of **(A)** Dex or **(B)** MPA. Induction is shown as percentage luciferase activity expressed in relative light units. Non-linear regression and sigmoidal dose response curves were used to analyse data. The inset shows the result from 0 to 100%. The result shown is the averages (±SEM) of two independent experiments with each condition performed in triplicate.

Table 3.2: Potencies for transactivation by GR ligands via overexpressed GR in the absence and presence of Vpr, expressed as EC_{50} in M, as well as efficacy (maximal agonist activity expressed as a percentage)[1].

Ligand	EC_{50} (M) ±SEM		MAX (%) ±SEM	
	-Vpr	+Vpr	-Vpr	+Vpr
Dex	$1.45 \times 10^{-8} \pm 2.01 \times 10^{-8}$	$6.96 \times 10^{-7} \pm 6,13 \times 10^{-7}$	100 ± 0.00	728.12 ± 41.78
MPA	$1.14 \times 10^{-8} \pm 2.72 \times 10^{-9}$	$1.87 \times 10^{-8} \pm 1.26 \times 10^{-8}$	118.48 ± 7.48	195.94 ± 18.99

[1]Data from experiments depicted in Figure 3.12 were analysed to obtain $EC_{50} \pm$ SEM values, as well as maximal (MAX) values ± SEM for each test compound in the absence and presence of Vpr. Maximal values are expressed as a percentage of 10^{-5} M Dex -Vpr = 100%. Statistical analysis of the EC_{50} values for agonist activity, indicated Dex -Vpr vs. Dex +VPR (p<0.05); MPA -Vpr vs. MPA +Vpr (p>0.05), whereas the statistically significant differences observed for the MAX values indicated that Dex -Vpr vs. Dex +Vpr (p<0.01) and MPA -Vpr vs MPA +Vpr (p<0.05).

CHAPTER 4 : DISCUSSION AND CONCLUSION

4.1 Discussion

Although the synthetic progestins MPA and Net-EN/Net-A are widely used in both contraception and HRT, little is known about their effects on the local immunity in the vaginal environment, a crucial site for HIV-1 infection during male to female transmission of HIV (132). Recent clinical evidence indicating that the use of MPA in contraception increases the risk of HIV-1 transmission and infectivity (39,44), has highlighted the importance of understanding how factors, such as hormonal contraception, may influence the local mucosal immune response in the human female genital tract (59). It is known that inflammation of the lower female genital tract increases susceptibility to HIV-1 (133), and that the mucosal immune function is modulated when an excess of pro-inflammatory cytokines are released (134). In this study, it was investigated whether MPA and Net-A, in comparison to P4, could modulate the local immune response in the vaginal mucosa, by investigating the regulation of pro-inflammatory chemokine gene expression in response to these ligands. Chemokines are chemotactic cytokines that elicit an inflammatory reaction by activating and recruiting leukocytes to the site of injury or infection (38).

An epithelial cell line derived from the human vaginal mucosa (Vk2/E6E7) was used as an *in vitro* model to investigate the effect of synthetic progestins on vaginal mucosal immunity. The Vk2/E6E7 cell line displays characteristics of stratified squamous nonkeratinizing epithelia, and constitutively express the chemokine MIP-1β (55,56). Furthermore, production of MIP-1β protein in the Vk2/E6E7 cells increased when the cells were treated with TNF-α (56). Fichorova *et al.* were unable to detect MIP-1α protein in the Vk2/E6E7 cell line in the absence and presence of TNF-α (55).

4.1.1 Differential MIP-1α and MIP-1β regulation by P4, MPA, and Net-A

Since the expression of MIP-1α and MIP-1β have been shown to vary or be expressed at very low basal levels in the Vk2/E6E7 cell line, the first objective was to determine whether MIP-1α and MIP-1β mRNA were expressed in the Vk2/E6E7 cells, basally and with TNF-α treatment. The cells were treated with TNF-α for various time periods to determine the optimal time for the expression of mRNA of these chemokines. Using conventional PCR and QPCR, the results showed no basal expression of MIP-1α or MIP-1β. Furthermore, the optimal TNF-α-induced expression of both MIP-1α and MIP-1β was found to be two hours (Figure 3.1 and Figure 3.2). All further experiments, investigating MIP-1α and MIP-1β gene expression in the Vk2/E6E7 cell line, were thus performed at two hours.

The general trend that was observed for the effect of P4, MPA and Net-A on MIP-1α and MIP-1β mRNA expression is that P4 and MPA upregulate the expression of both genes in the Vk2/E6E7 cell line, while Net-A has no effect (Figure 3.3A and 3.3B). Both genes were significantly upregulated by P4, while the upregulation by MPA was significant only for MIP-1β. Similar P4-induced increases in the expression of MIP-1α and MIP-1β at the protein level have previously been shown in the epithelial layer of the female genital tract during the secretory phase of the menstrual cycle, when serum P4 levels are high (64,135). However, the effect of P4 on the secretion of these chemokines appear to be cell specific. Vassiliadou *et al.* previously showed that P4 does not influence the secretion of MIP-1α or MIP-1β in CD4^{+} cells, but downregulates the secretion of these chemokines in CD8^{+} cells (136). Similar cell specific effects on MIP-1β secretion have been observed with MPA. The results from this study show that MPA upregulates both MIP-1α and MIP-1β gene expression in the Vk2/E6E7 cell line, while MPA has no effect on MIP-1α or MIP-1β secretion in human endometrial glandular epithelial primary cells or endometrial stromal primary

cells (137).

Interestingly, neither the expression of MIP-1α nor MIP-1β genes was regulated by Net-A. To the best of the author's knowledge, there are no previous studies showing the regulation of MIP-1α and MIP-1β gene expression by Net-A. However, Africander *et al.* showed that Net-A also has no effect on the expression of another β-chemokine, RANTES, in the Vk2/E6E7 cell line (11). Taken together, the results show that MPA and Net-A have differential effects on the mRNA expression of MIP-1β, and possibly MIP-1α, and that these responses may differ from those of P4. It was hypothesized that the apparent ligand-specific effects on the expression of these genes in the vaginal epithelial cells, are due to the ligands acting via different steroid receptors.

4.1.2 *The ER is expressed in the human vaginal epithelial cell line (Vk2/E6E7)*

The levels of PR, GR, AR and MR in the Vk2/E6E7 cell line had previously been determined (13). However, the levels of the ER, another member of the steroid receptor family, were not evaluated. In Section 3.3, it was shown that the Vk2/E6E7 cell line contains approximately 7.12 $f$mol/mg ER, and these levels are similar to those of the AR and GR in the Vk2/E6E7 cell line ($p>0.05$), but significantly less than the PR ($p<0.001$), [(11); Addendum F]. Furthermore, Western blot analysis showed that both ER isoforms, ERα and ERβ, are expressed in the Vk2/E6E7 cells (Figure 3.4). Consistent with the results of this study, vaginal tissue has been shown to express both ER isoforms (138). The levels of expression vary depending on the woman's age as well as hormone levels (138). However, since the majority of the literature indicates that P4, MPA and Net-A do not bind to the ER (82,126,127,139), it is unlikely that the effects of MPA on MIP-1α and MIP-1β gene expression are mediated via the ER.

4.1.3   A role for the GR in MIP-1α gene regulation by P4 and MPA

A recent review on the mechanism of action of synthetic progestins via steroid receptors, summarises the evidence that although P4, MPA and Net-A can bind to the GR, only P4 and MPA have been shown to display partial glucocorticoid agonist activity (81). Since P4 and MPA, but not Net-A, can upregulate the expression of MIP-1α and MIP-1β in the Vk2/E6E7 cells, it is likely that the GR mediates these effects. Results from Western blot analysis (Figure 3.5A) and promoter reporter assays (Figure 3.5B) confirmed the previously reported expression and functional activity of the endogenous GR in the Vk2/E6E7 cells (13).

From the results shown in Figure 3.7A, the reduction in the GR protein levels (~40%) appeared to abrogate the MPA-induced upregulation of MIP-1α mRNA expression, while only partially inhibiting the P4-induced effects. In contrast, neither P4 nor MPA-induced effects on MIP-1β gene expression were GR-mediated (Figure 3.7B). The Dex-mediated repression of MIP-1α gene expression, as well as the Dex-mediated upregulation of MIP-1β gene expression, appeared to be negated by the silencing of the GR. This suggests the involvement of the GR in mediating the effects of the GR-specific ligand on both these genes in the Vk2/E6E7 cell line (Figure 3.7A and 3.7B). Dex has previously been shown to upregulate the anti-IgE-stimulated expression of both MIP-1α and MIP-1β in human mast cells (140), while repressing lipopolysaccharides (LPS)-induced expression of MIP-1α in rat lung (141), suggesting that the effects of Dex on the expression of MIP-1α is cell-specific. The differential effects of Dex on MIP-1α and MIP-1β mRNA expression via the GR in the same cell line were surprising.

In an attempt to explain the differential responses of Dex on MIP-1α and MIP-1β mRNA expression, the general mechanism of action of the GR should be considered. As described in Section 1.4.1, the GR can directly bind to GRE binding sites, which leads to the activation of gene

transcription, or tether to transcription factors, such as NFκB, to repress gene transcription. However, these are complex interactions and thus predicting the biological outcome when a ligand binds to the GR is not simplistic. The outcome is dependent on the promoter architecture of the gene, particularly the nature, number and accessibility of *cis*-regulatory elements. In addition, gene regulation occurs in the context of chromatin, and accessibility of regulatory elements for transcription factors such as the GR, can be blocked by nucleosomes (reviewed in 142). Human MIP-1α and MIP-1β promoters contain binding sites for several transcription factors, such as CCAAT/enhancer binding protein (C/EBP), NFκB and AP1 (140,143–145). Interestingly, there are more NFκB binding sites in the MIP-1α promoter than in the MIP-1β promoter (144–146). Furthermore, MIP-1β contains four putative GRE sites in proximity of the promoter, while MIP-1α contains only two putative GRE sites (140). Taking into account the fact that MIP-1β has more GREs for activation of transcription, and MIP-1α has more NFκB sites for repression of transcription, it is possible that Dex could increase the expression of MIP-1β via the binding of the GR to one or more GREs, while Dex could decrease the expression of MIP-1α via tethering of the GR to NFκB or AP1.

The upregulation of MIP-1α mRNA expression, in response to P4 and MPA, is most likely via the recruitment of the GR to GRE binding sites in the promoter of the MIP-1α gene. Considering that MPA has previously been shown to have a slightly greater efficacy for transactivation via the GR than P4 (34) it is surprising that P4 upregulates MIP-1α expression to a greater extent than MPA. One possible explanation for the differences observed in this study versus the observations by Koubovec *et al.* (34), could be the use of different cell types as well as different promoters. This study used Vk2/E6E7 cells with endogenously expressed steroid receptors and promoters, while Koubovec *et al.* (34) used HEK293 cells stably transfected with only the GR and a synthetic minimal GRE-containing promoter. Thus, it is possible that in the Vk2/E6E7 cells, the effect of P4

on MIP-1α mRNA expression is partially mediated via another steroid receptor, such as the PR, or that the MPA-bound GR has less access to *cis*-regulatory elements in the promoter of MIP-1α than the P4-bound GR.

Although P4 and MPA exhibited similar effects on both MIP-1α and MIP-1β gene expression, only the regulation of P4 and MPA on MIP-1α gene expression appeared to be GR-mediated, suggesting that similar biological responses are being elicited by different steroid receptors on two related genes in the Vk2/E6E7 cells. Considering that both the PR and AR are expressed in this cell line, and that both can bind GREs (reviewed in 74), as well as the fact that both P4 and MPA have been reported to exhibit agonist activity via the PR and AR (84,147)t is possible that either of these receptors may be involved. As Net-A has previously been shown to have similar androgenic and progestagenic activities to that of MPA (82–84), it would be expected that Net-A displays similar activity to MPA. However, Net-A did not show any activity on these genes. It may be that Net-A is metabolised in the Vk2/E6E7 cell line and these metabolites have less progestagenic or androgenic activity than Net-A. Similar differential effects of MPA and Net-A were recently shown on the RANTES gene in a human ectocervical epithelial cell line (Ect1/E6E7) (11). Africander *et al.* (11) showed the repressive effects of MPA were mediated by the AR, while Net-A had no effect. In addition, and rather unexpectedly, when the GR levels were reduced, treatment with Net-A caused an upregulation of the MIP-1β gene expression, suggesting that the GR may inhibit Net-A-induced expression of MIP-1β, possibly via a steroid receptor other than the GR.

4.1.4   *Modulation of ligand-induced MIP-1α gene expression by the HIV-1 accessory protein, Vpr*

Vpr has been shown to modulate GR activity, and has also been associated with the regulation of host gene expression, including chemokines such as MIP-1α and MIP-1β (108–110,116,118). As the

results from this study implicate the GR in mediating the effects of P4 and MPA on MIP-1α gene expression (Figure 3.7), it was next investigated whether overexpressed Vpr could modulate the action of these GR agonists on endogenous MIP-1α expression in the Vk2/E6E7 cell line. The results show that Vpr negates the P4-, Dex-, and possibly the MPA-induced effects on MIP-1α expression (Figure 3.8). Interestingly, Vpr had no effect on MIP-1α mRNA expression in the presence of Net-A, previously shown to have no GR agonist activity. Thus, these results indicate that Vpr modulates the activity of GR agonists on MIP-1α expression. A limitation of this study was that the commercial antibody detecting the hemagglutinin epitope (HA) tag of the transfected Vpr could not be optimized and thus the expression of Vpr could not be shown by Western blotting. To investigate whether the effects of Vpr on MIP-1α are GR-dependent, the GR levels were reduced by siRNA (Figure 3.9). The reduction of GR protein inhibited the actions of Vpr on P4 and MPA-induced MIP-1α expression (Figure 3.10). Furthermore, the results indicated that silencing of the GR, not only abrogated Vpr effects in the presence of P4, but also repressed MIP-1α gene expression extensively. These results are difficult to interpret, especially considering that the P4 and MPA results are from a single experiment, as time did not allow further repeats of this experiment. Figure 4.1 represents a model suggesting a possible interpretation of these results. Briefly, P4 and MPA can both activate transcription of the MIP-1α gene via the GR binding to a GRE, followed by the recruitment of cofactors (Panel A+C). Vpr can abolish these effects possibly due to it competing with cofactors for binding to the GR, thereby preventing transcription of the MIP-1α gene (Panel B). When Vpr is present, but the GR is absent, MPA has no effect on MIP-1α gene expression, while P4 binds to another steroid receptor, possibly the PR, causing repression of the gene (Panel D).

Surprisingly the Dex-induced repression on MIP-1α, in the presence of Vpr, did not change when the GR was knocked down. Taken together the results suggest that Vpr modulates the repression of

P4 and MPA-liganded GR, but not the Dex-liganded GR, on MIP-1α mRNA expression. Interestingly, Mirani *et al.* (92) have previously shown that the GR-dependent Dex repression of the pro-inflammatory cytokine, IL-12, was further repressed by Vpr in PBMCs. Thus, the ability of Vpr to mediate gene expression via the GR may be gene-specific, ligand-specific and/or cell-specific.

Taken together, Vpr suppresses the P4- and MPA-induced expression of both MIP-1α and MIP-1β genes in the human vaginal epithelial cell line. This suppression by Vpr appear to be GR-dependent. Furthermore, the repression of MIP-1α gene expression by Vpr observed in the presence of P4 is likely via the PR. Indeed, Vpr has been reported to interact with the PR and the ER (95). As different cells express varying steroid receptors, it may be that the effects of Vpr will be cell specific.
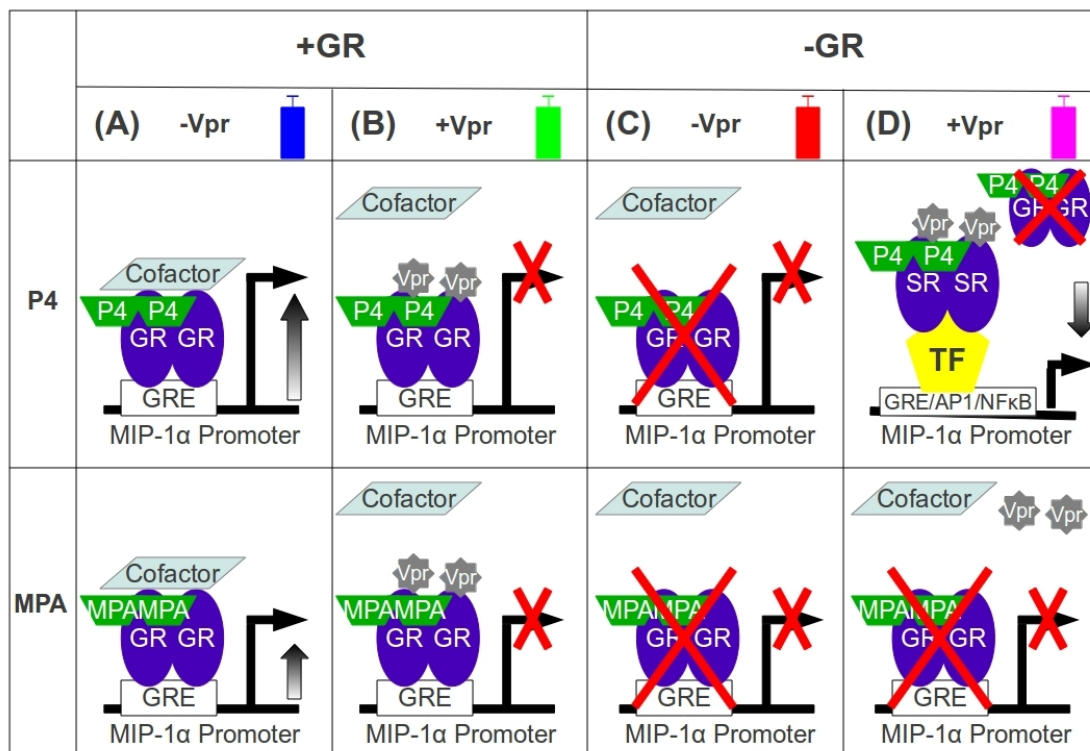
Figure 4.1: Proposed model summarizing the possible mechanism of action of P4 and MPA via the GR in the absence and presence of Vpr. Abbreviations: GR, glucocorticoid receptor; +GR, in the presence of endogenous GR; -GR, after the knockdown of endogenous GR; GRE, glucocorticoid response element; P4, progesterone; MIP-1α, macrophage inflammatory protein 1α; MPA, medroxyprogesterone acetate; SR, steroid receptor; TF, transcription factor; Vpr, viral protein R.

4.1.5   Vpr modulates Dex and MPA glucocorticoid agonist activity for transactivation on a reporter gene.

The effects of both Dex and MPA on the expression of the MIP-1α gene were shown to be most likely mediated via the GR. Additionally, Vpr's inhibition of the P4- and MPA-induced expression of the MIP-1α gene was shown to be GR-dependent. Next a promoter-reporter assay to test the effect of overexpressed Vpr on Dex and MPA agonist activity for transactivation on a reporter gene via overexpressed human GR, was performed in COS-1 cells. This cell line is an excellent model for comparing activities of ligands via a specific transiently transfected receptor, as these cells are devoid of endogenous steroid receptors. In the absence of Vpr, both Dex and MPA were shown to have similar efficacy and potency via the GR on the synthetic reporter gene (Figure 3.12). The efficacy of a ligand is defined as the maximal response that can be elicited by that ligand in a given cell, under specific experimental conditions (81). An agonist refers to a ligand that has a similar efficacy as the endogenous ligand, whereas a partial agonist is a ligand that has lower efficacy than that of the agonist, and a supra-agonist is a ligand that has an efficacy higher than the efficacy of the agonist (81). Potency of a ligand is a measure of the concentration of ligand needed to elicit half the maximal response, and commonly quantified as the EC_{50} (81). Based on these definitions the results from this study show that both Dex and MPA are GR agonists. In contrast, Koubovec *et al.* (34) have previously shown, in HEK293 cells stably transfected with GR, that MPA is a partial agonist via the GR, while Dex is an agonist. Furthermore, they showed that Dex is more potent than MPA. Similar results were obtained in COS-1 cells transiently transfected with the GR and a synthetic minimal promoter (67) These observed differences between this study and that of Koubovec *et al.* (34) and Ronacher *et al. (67)* could be due to the differences in the GR levels. It has previously been shown that receptor levels affect the potency and efficacy of full GR agonists, partial agonists and antagonists (148). Interestingly, Ronacher *et al.* (67) transfected more than 10 times the amount

of GR expression plasmid transfected in this study. In addition the lower potency of Dex and MPA in this study, also suggests that the GR levels are lower than in the above mentioned studies. The results of the present study suggest that Vpr may increase the maximal response that can be elicited by MPA and Dex on a reporter gene via the GR, changing both Dex and MPA from full agonists to supra-agonists. It is noteworthy that these results on a synthetic promoter-reporter differ from the Vpr results on the endogenous MIP-1α gene. It is possible that promoter-reporters may not reflect the regulation on full-length endogenous promoters in the context of the native chromatin structure.

The results showed that Vpr significantly increased the efficacies of both Dex and, to a lesser extent, MPA via the GR (Figure 3.12). Vpr has previously been shown to increase Dex efficacy via the GR on a synthetic GRE-promoter in the human embryonic kidney cell line, 293T (97). Consistent with this, Kino *et al.* (95) showed an increase in Dex efficacy in the presence of Vpr on a MMTV promoter-reporter construct in the human rhabdomyosarcoma cell line, A204. Interestingly, Vpr affects only the potency of the GR-specific agonist, Dex, but not that of MPA (Figure 3.12).

Taken together, Vpr modulates the efficacy of both Dex and MPA for transactivation on a synthetic GRE-containing, promoter-reporter gene via the GR, while changing the potency of only the GR specific ligand, Dex.

4.2   Conclusion and future research

Inflammation in the female genital tract increases susceptibility of the female genital tract to viral infections such as HIV (149,150). To date, very little is known about the relative effects of MPA and Net-A on local epithelial immune function of the vaginal mucosa. However, a number of studies have indicated increased susceptibility to viral and bacterial infections in response to MPA in the

female genital tract of both humans and primates (48,151–153).

The results from this study presented in Chapter 3, showed ligand-specific regulation of MIP-1α and MIP-1β gene expression in the vaginal epithelial cell line, Vk2/E6E7. Chemokines play a critical role in inflammation by activating human granulocytes (neutrophils, eosinophils and basophils) and recruiting leukocytes to the site of infection (154). The proinflammatory cytokines, MIP-1α and MIP-1β, play an important role in inflammation and have been linked to HIV-1 infection (155). The results suggest that P4 and MPA play a pro-inflammatory role in the vaginal epithelial cells, while Net-A is neither pro- or anti-inflammatory.

The physiological relevance of the P4-induced increase in the expression of the chemokine genes, in the Vk2/E6E7 cells, is not clear. As P4 has previously been shown to have a protective role in the vagina against the infection by pathogens (13), the results may indicate that P4, and to a lesser extent MPA, could be protective against infections in the vaginal mucosa. The fact that Net-A has no effect on either MIP-1α or MIP-1β may suggest that it does not provide the same protective effects as P4 and MPA. In contrast, it could be that Net-A, unlike P4 and MPA, would not cause chronic inflammation in the vaginal mucosa, which may be positive in terms of HIV-1 infection as an increase in inflammation has been shown to increase HIV-1 replication (reviewed in 156). Thus the role of an increase in inflammation is not clear since it is both protective, in the sense that is protects against the infection by pathogens and also non-protective since it would recruit CD4$^+$ cells that might become infected by HIV-1.

The HIV-1 accessory protein, Vpr, has previously been shown to downregulate the expression of MIP-1α, MIP-1β and RANTES, thereby decreasing the recruitment of immune cells to the site of inflammation, weakening the defense against other infections, and inhibiting the antiviral activity against HIV-1 (117,118). The results in this study show that Vpr abrogates the effects of P4 and

MPA on both MIP-1α and MIP-1β. If the upregulation of these genes by P4 and MPA are protective, then this means that Vpr eliminates these protective effects.

In terms of understanding the molecular mechanism of action of these ligands on immunity in the vaginal mucosa, a number of additional experiments can be performed. For example, the recruitment of the GR to specific cis-regulatory elements, such as GRE, NFκB, AP1 and C/EBP, found in the promoter region of the MIP-1α gene could be investigated by chromatin immunoprecipitation (ChIP) assays. This assay uses primers to specific *cis*-elements in the promoter of the MIP-1α gene, thus allowing the *cis*-element associating with the GR to be identified. Similar experiments could be performed to investigate the regulation of MIP-1β, once the steroid receptors mediating the effects of P4 and MPA have been identified using for a combination of siRNA technology or receptor specific antagonists.

Furthermore, the effects of the ligands on MIP-1α and MIP-1β protein production should be investigated as the results shown at the mRNA level may differ from those shown at the protein level due to post transcriptional regulation. This could be done by using either enzyme-linked immunosorbent assay (ELISA) or a Multiplex bead array. Antibodies specific for the MIP-1α and MIP-1β protein can be used to detect the production as well as the secretion of these chemokines in an ELISA. The multiplex bead array technology allows the simultaneous cytometric quantitation of multiple soluble cytokines and chemokines.

To gain more insight into the physiological relevance of this study, it would be of interest to examine the effects of P4, MPA and also Net-A on HIV-1 infectivity in the Vk2/E6E7 cells. Zheng *et al.* have previously shown that these cells can be infected by HIV-1 (157) and that they resemble their tissue of origin, as well as those of primary cultures (158), the effects on HIV-1 infectivity should ideally be investigated in primary vaginal cells or tissue explants. Moreover, it would be

interesting to investigate the expression of MIP-1α and MIP-1β in vaginal samples from healthy vs. HIV-1-infected women using MPA and Net-A as contraception, as compared to women not using these contraceptives.

To investigate whether modulation of host gene expression by Vpr in response to P4, MPA and Dex is mediated via a direct interaction between GR and Vpr, ChIP, as well as ChIP-reChip assays could be performed. The ChIP-reChIP assay is a direct strategy to determine the *in vivo* co-localisation of proteins interacting or in close contact in a chromatinized template. Thus the result from the ChIP-reChIP assay may indicate whether the GR and Vpr co-localise on the promoter regions of MIP-1α, in response to Dex, P4 and MPA. Another technique that may shed light on a direct interaction between the GR and Vpr is fluorescence resonance energy transfer (FRET) analysis using GR and Vpr tagged with the FRET couple cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP).

Dose response analyses are used to characterise the biological effect of ligands via receptors, by determining efficacy and potency. The result obtained when investigating efficacy and potency for transactivation via the GR on a synthetic reporter gene, in the absence of Vpr, differs from that reported in the literature (34) and may be due to differences in experimental conditions. However, similar to the literature, this study shows that Vpr increased the efficacy of the liganded GR (92,95,97,101,131). A limitation of this experiment, is that only two experiments were performed, with large errors between experiments.

In summary, the results presented in this thesis showed that P4, MPA and Net-A differentially regulate MIP-1α and MIP-1β gene expression in a human vaginal epithelial cell line. P4 and MPA upregulate MIP-1α and MIP-1β gene expression, while Net-A has no effect. Taking into account that both MPA and Net-A were designed to mimic the biological activity of P4, this result implies

that MPA has similar biological activity as natural P4 on these genes, while Net-A does not. As P4 has previously been shown to have a protective role in the vagina, it may imply that MPA is also protective, while Net-A is not. It was further shown that the upregulation of MIP-1α gene expression by P4 and MPA in the Vk2/E6E7 cells is mediated by the GR. This thesis also showed that Vpr inhibits the P4- and MPA-induced expression of MIP-1α and MIP-1β, and suggests that steroid receptors such as the GR and PR, are needed for the effects of Vpr. Finally, the results may further our understanding of the impact of the use MPA and Net-A, on the immune function of the female genital tract *in vivo* and the possible implications for HIV-1 infection.

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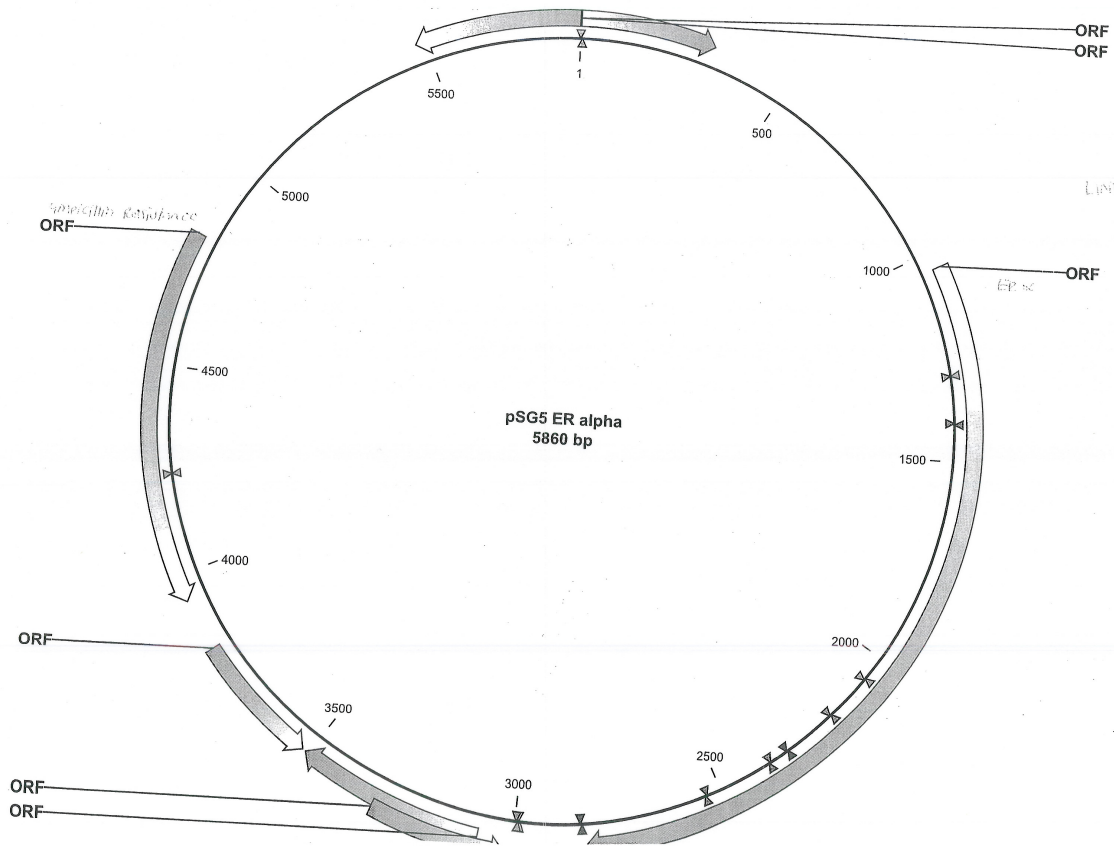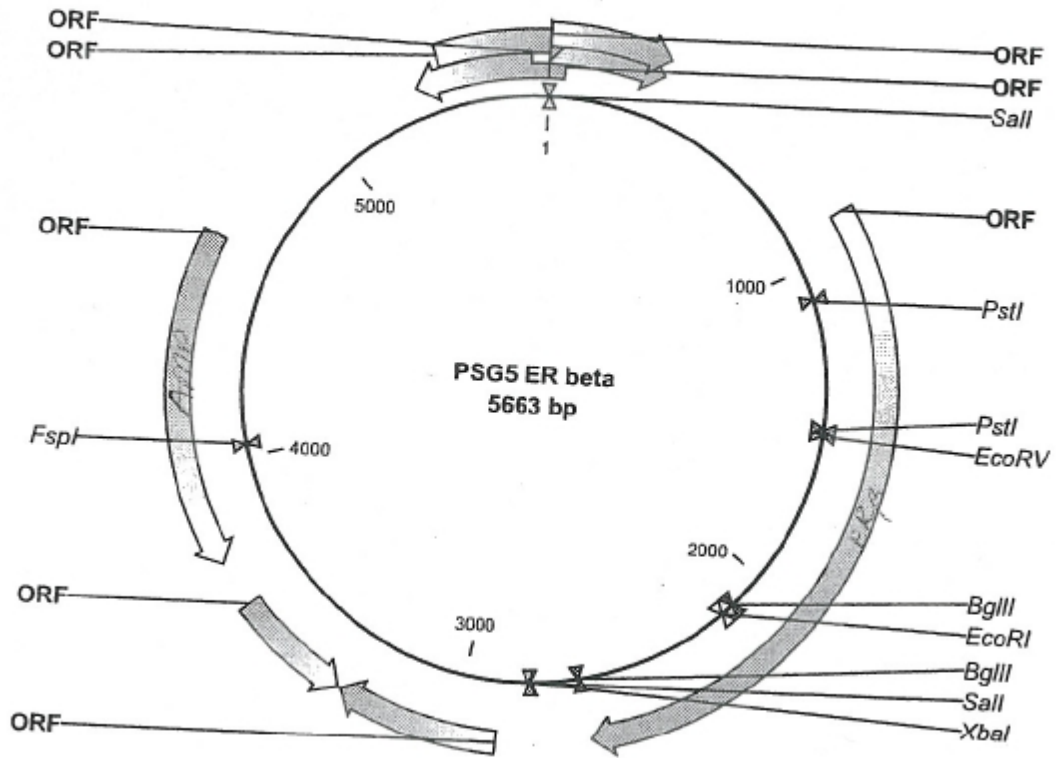
ADDENDUM A: PLASMID MAPS

A1 List of Plasmids

Table A1.1: Plasmids used during the study, including the references and the source of the plasmid.

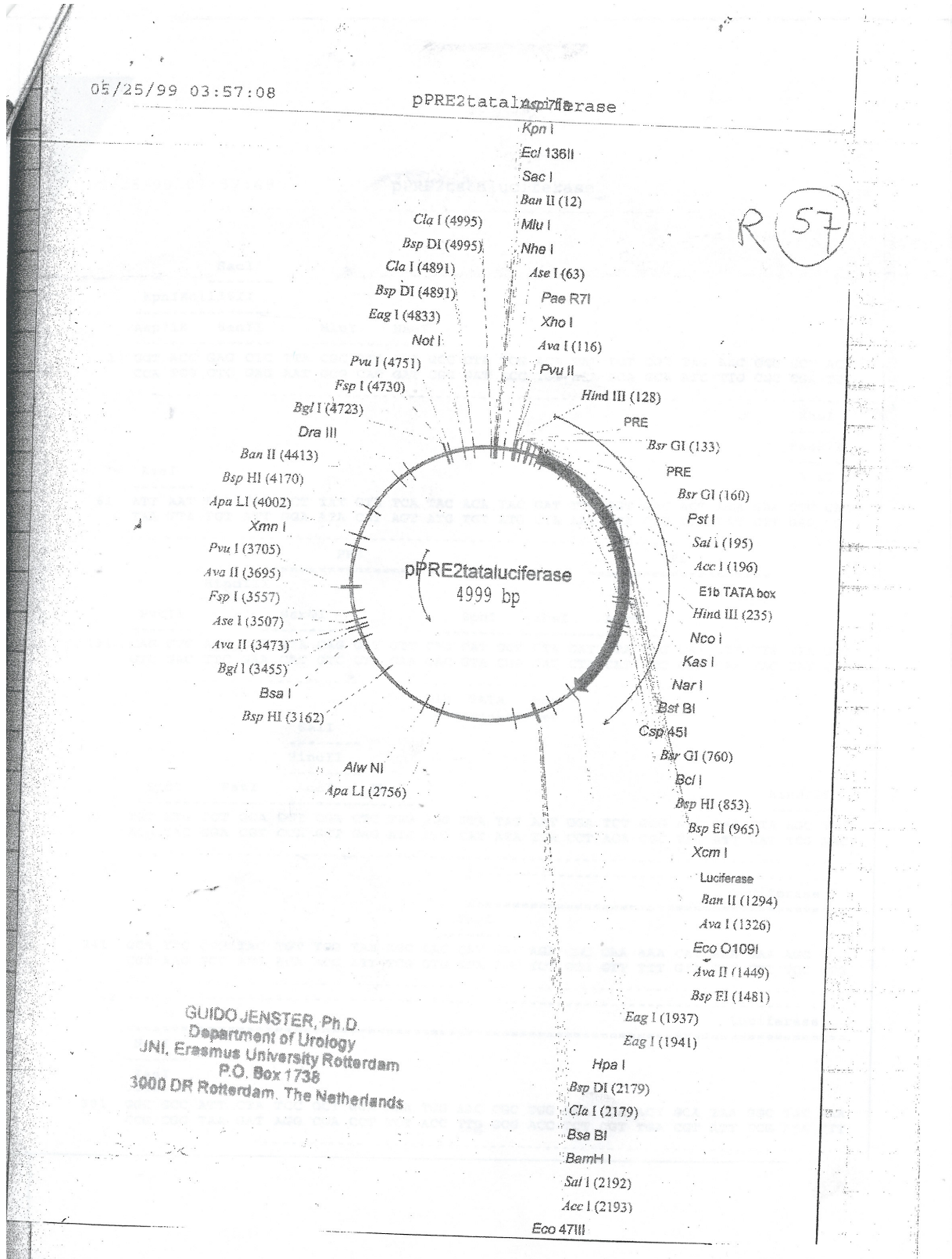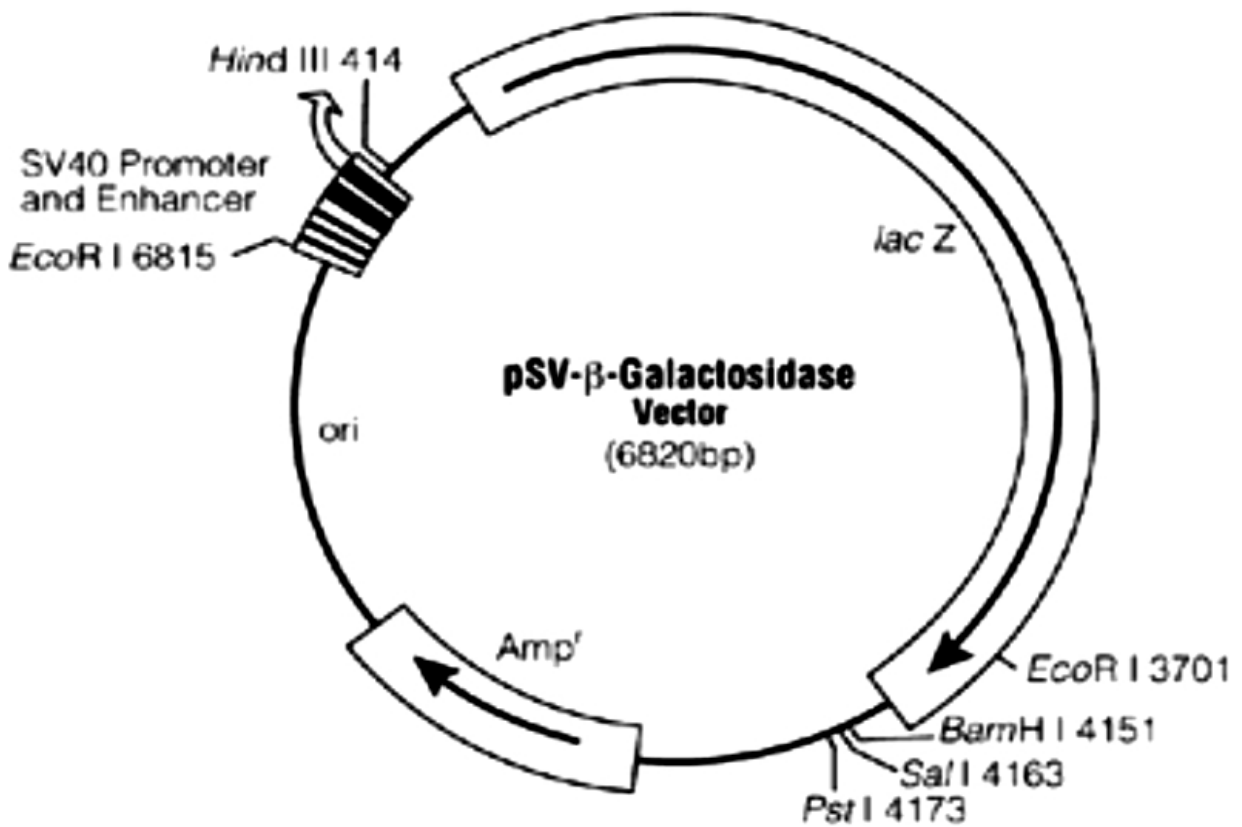| Plasmid Name	Vector	Vector + Insert Size (kb)	References	Source
pRShGRα	pRS	6.7	(159)	R. M. Evans (Howard Hughes Medical Institute, La Jolla, United States of America)
pSG5ERα	pSG5	5.9	(160)	F. Gannon (Europian Molecular Biology Laboratory, Heidelberg, Germany)
pSG5ERβ	pSG5	5.7	(161)	F. Gannon (Europian Molecular Biology Laboratory, Heidelberg, Germany)
p-TAT-GRE-E1b-Luc	Promoter E1b	5.0	(162)	G. Jenster (Erasmus University of Rotterdam, Netherlands)
pSV-βgal	pSV	6.8	(163)	Promega Corporation, Wyoming, United States of America
pGL2-Basic	pGL2	5.6	(164)	Promega Corporation, Wyoming, United States of America
pCMV4-HA·Vpr	pCMV4	5.2	(97)	W. Greene (Gladstone Institute of Virology and Immunology, University of California, San Francisco, United States of America)

A2 Plasmid Maps

A2.1 pRShGRα expression vector

A2.2 pSG5ERα expression vector

A2.3 pSG5ERβ expression vector

A2.4 p-TAT-GRE-E1b-luciferase reporter vector

05/25/99 03:57:08   pPRE2tataluciferase

R 57

pPRE2tataluciferase
4999 bp

Kpn I
Ecl 136II
Sac I
Ban II (12)
Mlu I
Nhe I
Ase I (63)
Pae R7I
Xho I
Ava I (116)
Pvu II
Hind III (128)
PRE
Bsr GI (133)
PRE
Bsr GI (160)
Pst I
Sal I (195)
Acc I (196)
E1b TATA box
Hind III (235)
Nco I
Kas I
Nar I
Bst BI
Csp 45I
Bsr GI (760)
Bcl I
Bsp HI (853)
Bsp EI (965)
Xcm I
Luciferase
Ban II (1294)
Ava I (1326)
Eco O109I
Ava II (1449)
Bsp EI (1481)
Eag I (1937)
Eag I (1941)
Hpa I
Bsp DI (2179)
Cla I (2179)
Bsa BI
BamH I
Sal I (2192)
Acc I (2193)
Eco 47III

Cla I (4995)
Bsp DI (4995)
Cla I (4891)
Bsp DI (4891)
Eag I (4833)
Not I
Pvu I (4751)
Fsp I (4730)
Bgl I (4723)
Dra III
Ban II (4413)
Bsp HI (4170)
Apa LI (4002)
Xmn I
Pvu I (3705)
Ava II (3695)
Fsp I (3557)
Ase I (3507)
Ava II (3473)
Bgl I (3455)
Bsa I
Bsp HI (3162)

Alw NI
Apa LI (2756)

GUIDO JENSTER, Ph.D.
Department of Urology
JNI, Erasmus University Rotterdam
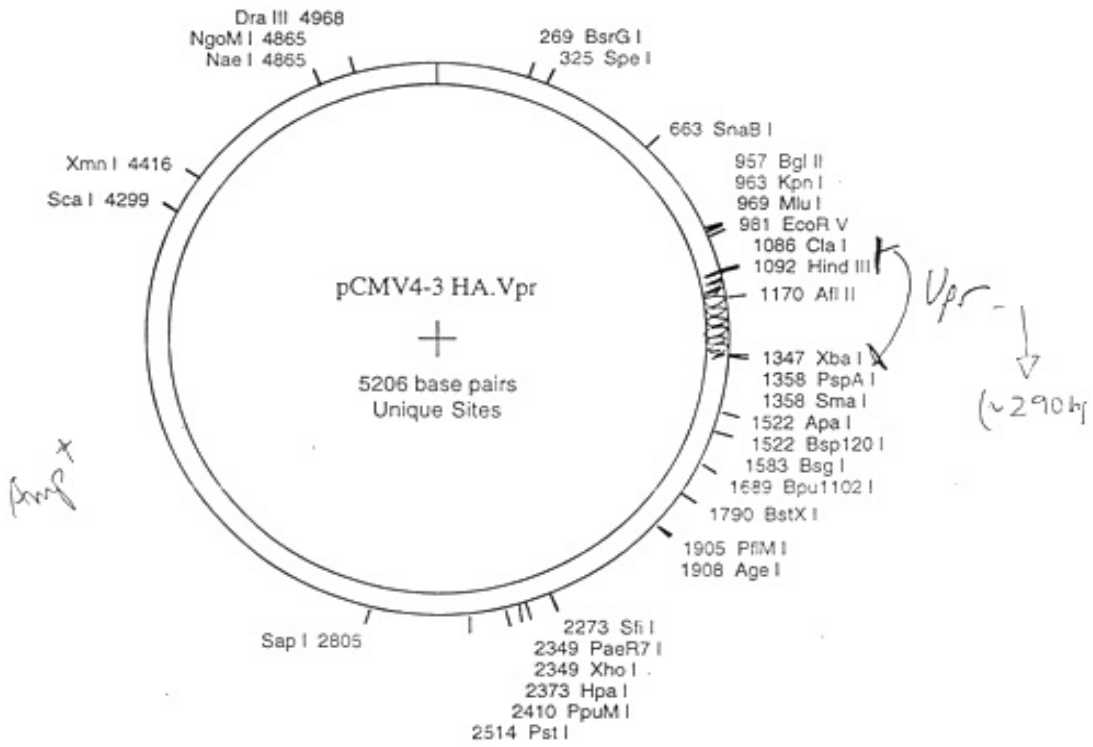P.O. Box 1738
3000 DR Rotterdam, The Netherlands

A2.5 pSV-β-galactosidase expression vector used to control for transfection efficiency

A2.6 pGL2-Basic expression vector and multiple cloning sequences
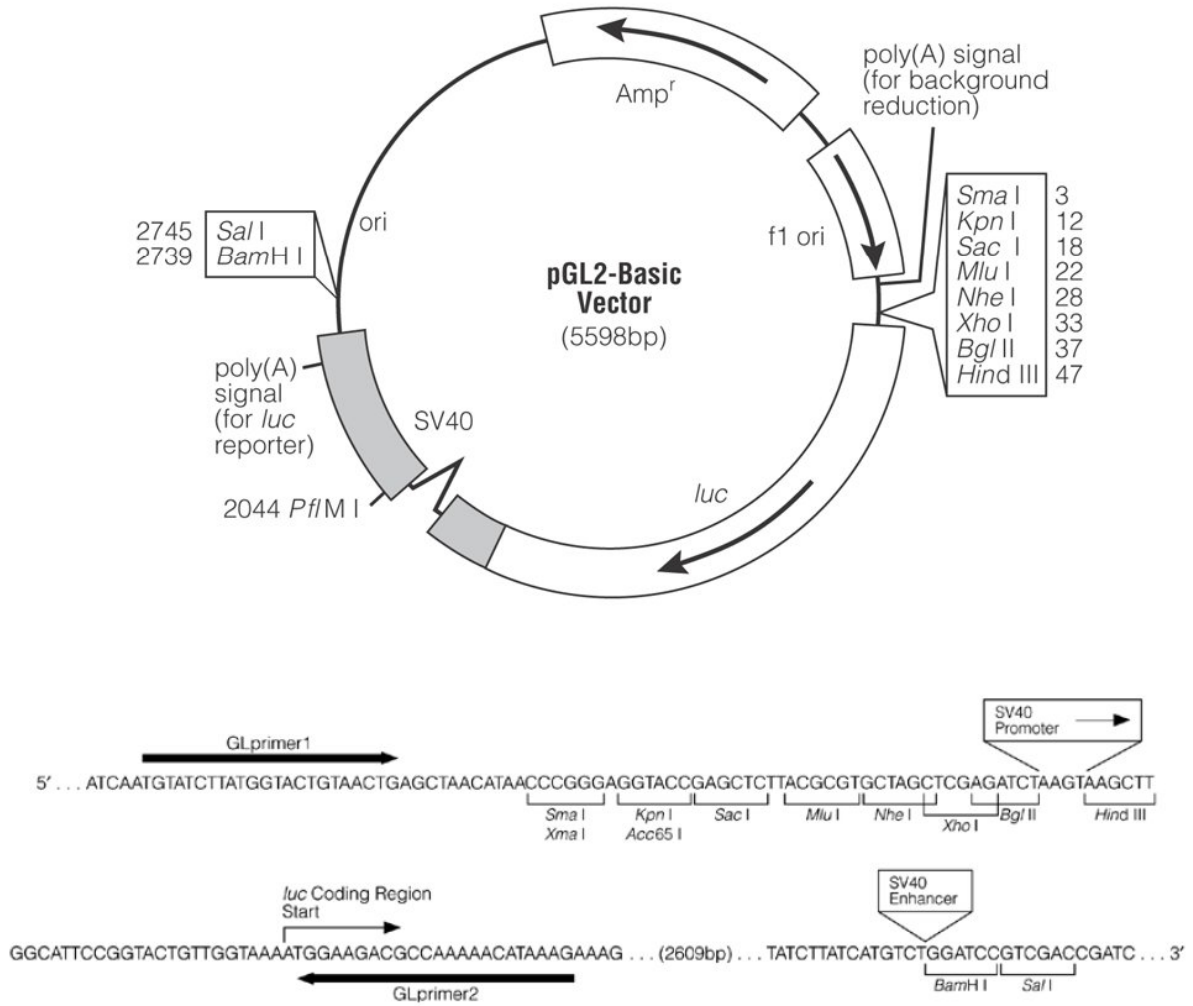
A2.7 pCMV4-HA·Vpr

ADDENDUM B: ANTIBODIES AND PCR AGAROSE GELS

B1 List of Antibodies and optimal dilutions

Table B1: A list of antibodies used for Western blot analysis[1].

Primary Antibody	Catalogue Number	Dilution	Secondary Antibody	Catalogue Number	Dilution
rabbit-anti-GRα (H300)	sc-8992	1:4000	goat-anti-rabbit	sc-2030	1:10000
mouse-anti-AR (411)	sc-7305	1:1000	goat-anti-mouse	sc-2005	1:5000
rabbit-anti-ERα (MC-20)	sc-542	1:200	goat-anti-rabbit	sc-2030	1:1000
rabbit-anti-ERβ (H-150)	sc-8974	1:1000	goat-anti-rabbit	sc-2030	1:5000
mouse-anti-GAPDH (0411)	sc-47724	1:6000	goat-anti-mouse	sc-2005	1:5000

[1]All antibodies were obtained from Santa Cruz Biotechnology, USA.
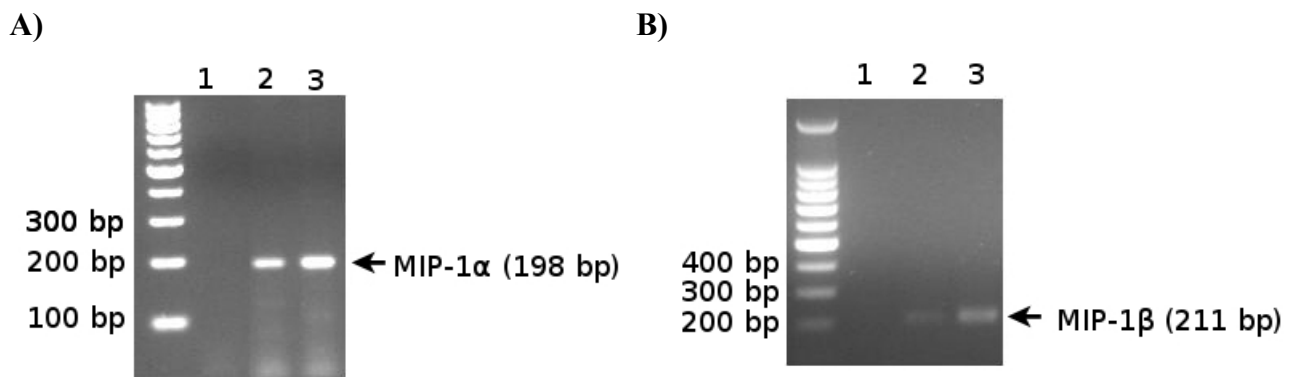
B2 Conventional PCR representative gels

Figure B2.1: Representative gels of conventional PCR products of (A) MIP-1α and (B) MIP-1β. Vk2/E6E7 cells were treated with 0.1% (v/v) EtOH in the absence and presence of 0.02 μg/ml TNF-α for 2h. Total RNA was isolated, cDNA synthesized, and analysed using conventional PCR with primers specific to MIP-1α **(A)** and MIP-1β **(B)**. Products were electrophoresed on a 2% agarose gel and visualised using etidium bromide staining. Lane 1 represents the no template control and lane 2, a sample induced with 0.1% (v/v) EtOH. Lanes 3 represents the PCR amplicons after treatment with 0.1% (v/v) EtOH in the presence of 0.02 μg/ml TNF-α for 2h.
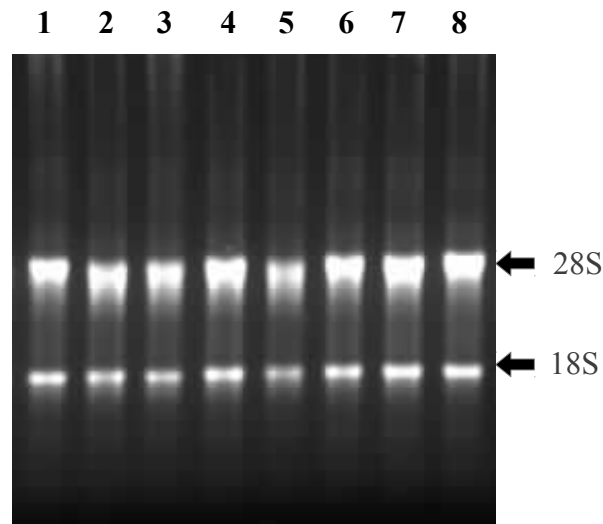
ADDENDUM C: QUANTITATIVE REALTIME PCR

Figure C1: A representative agarose showing the integrity of RNA harvested from Vk2/E6E7 cells. Vk2/E6E7 cells were treated with 0.02 μg/ml TNF-α and 1μM EtOH, P4, MPA and Net-A in the absence (Lane 1, 2, 3 and 4) and presence (Lane 5, 6, 7 and 8) of VPR. Total RNA was isolated as described in section 2.6 of Chapter 2. A total of 1μg RNA from each sample was electrophoresed on a denaturing 1% agarose gel and visualised by ethidium bromide staining. A representative figure of at least five independent experiments is shown.

Realtime QPCR is a powerful, highly sensitive method that allows for the detection and quantification of low abundance transcripts, as well as small changes in gene expression (122). The simplest detection method in QPCR uses SYBR Green I fluorescent dye, which binds to the minor groove of double stranded DNA (165). The use of fluorescent dyes during QPCR allows for the simultaneous PCR amplification, detection of the double stranded DNA product, the quantification of new double stranded DNA being formed as well as verification of the product by a melting curve (122). When using this method, it is important to isolate high quality, intact RNA, as poor quality RNA can compromise experimental results. The RNA quality was thus assessed by two methods prior to reverse transcription. The first was determining the optical density (OD) at both 260 nm (specific for nucleic acids) and 280 nm (specific for proteins) wavelengths, using a spectrophotometer. Good quality RNA is considered to have an OD 260/280 ratio higher than 1.9

(166). As the 260 nm reading can be compromised by the presence of genomic DNA in the sample, RNA was also analysed on a denaturating formaldehyde agarose gel. A good quality sample shows two bands, a 28S and 18S RNA band, with the 28S band approximately twice the intensity of the 18S band. Genomic DNA generally does not migrate through the gel because of its size, while a smear could indicate degradation of RNA. Figure C1 is a representative gel showing intact RNA.

Table C1: Reaction mix for QPCR

Reaction mix for the genes	MIP-1α & β	GAPDH
Forward Primer (5 mM)	1 µl	1 µl
Reverse Primer (5 mM)	1 µl	1 µl
MgCl$_2$ (25 mM)	0.8 µl	1.2 µl
Taq (5 u/µl)	1 µl	1 µl
PCR H$_2$O	5.2 µl	4.8 µl
Total	10 µl	10 µl

Following the assessment of the RNA integrity, cDNA was synthesized as described in Chapter 2 (Section 2.7), followed by QPCR analysis (see Table C1 for QPCR reaction mix). Prior to calculating the relative expression of the genes of interest by the method of Pfaffl (122), the efficiency of primer pairs had to be determined. Generally, PCR efficiency is assumed to be two since the product is doubled with every PCR cycle. However, the PCR efficiency may vary as it is dependent on the primer efficiency (E) and the PCR reaction (reviewed in 167). Analysis of QPCR results using the Pfaffl method allows for these differences in primer efficiency to be taken into account, and thus an accurate analysis can be obtained. The primer efficiency for MIP-1α and MIP-1β was thus determined by making serial dilutions of a single cDNA sample, and performing triplicate QPCR reactions for each dilution. Using Roche LightCycler software, standard curves were generated by plotting the crossing points (CP) for each dilution (Y-axis) against the log of cDNA concentration (X-axis) as shown in Figure C2A and C2B.

As shown in Equation 1 (122) the slope of the curve can then be used to calculate E.

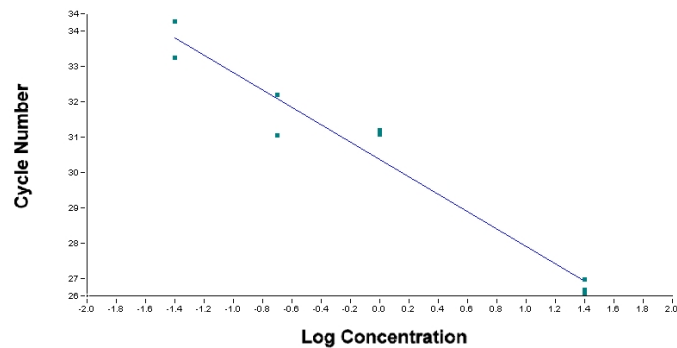$$Efficiency\,(E)=10^{(-1/slope)}$$ (Equation 1)

A)
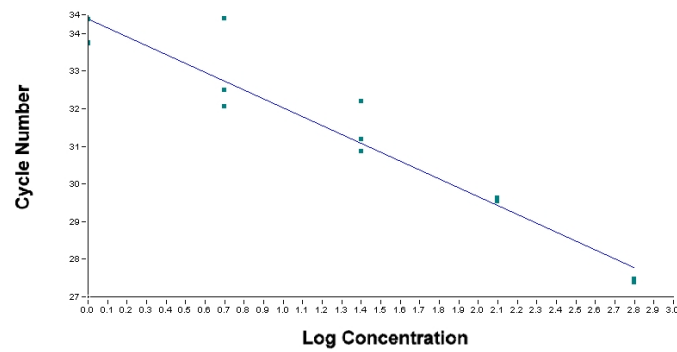
B)

Figure C2: Example of dilution series data generated by Lightcycler software for (A) MIP-1α and (B) MIP-1β amplification products.

The primer efficiencies as well as all other details of primer pairs used in this study are summarised in Table C2.

Table C2: Primers pairs for conventional PCR and QPCR[1]

Gene	Forward primer	Reverse Primer	Primer Efficiency	Reference
MIP-1α	TGCAACCAGTTCTCTGCATC	TTTCTGGACCCACTCCTCAC	2.52	(120)
MIP-1β	AAGCTCTGCGTGACTGTCCT	GCTTGCTTCTTTTGGTTTGG	2.54	(120)
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA	1.842	(121)

[1]The primer efficiency for GAPDH in the Vk2/E6E7 cell line was determined by Africander *et al.* [(11); Addendum F], and confirmed in this study.

Prior to determining the relative gene expression levels it is necessary to perform a melting curve analysis at the end of the PCR reaction. SYBR Green I complexes with all double stranded DNA products in the PCR reaction. This is due to the fact that the fluorescence measured during a QPCR using SYBR Green I, is the measurement of the sum of the relative levels of all products formed during the reaction. It is thus necessary to ensure that the measured fluorescence is of that of a single product. This is done by gradually increasing the temperature of the samples by 0.2ºC per second, until the denaturing temperature (95ºC) is reached. The position of the melting peak is unique to each product as it is dependent on the GC/AT ratio, the size and the sequence of the product (168). Thus one peak at high amplitude on the melting curve is indicative of one product, which is representative of the ideal melting curve. Additional peaks and shoulders may occur. This may indicate the presence of additional products such as primer dimers or non-specific products (168).

An example of a melting curve for MIP-1β is shown in Figure C3.

Once it had been determined that a single product had been amplified, the relative expression (R) of

86

the genes of interest could be determined by using equation 2 (122), and expressed relative to the expression levels of the untreated control.

$$Relative\ expression\ levels = \frac{(E_i)^{\Delta CP(control-sample)}}{(E_r)^{\Delta CP(control-sample)}}$$

(Equation 2)

E_i is the amplification efficiency of the gene of interest, while E_r refers to the efficiency of the reference gene. ΔCP is the difference of the crossing point between the vehicle control and the treated samples, calculated for the gene of interest and the reference gene (122).

The results of the experiments are graphically represented by plotting the expression levels of the genes of interest as a ratio of the reference gene (Example Figure 3.3A and B in Chapter 3). A ratio of one indicates no difference between the untreated and treated samples.
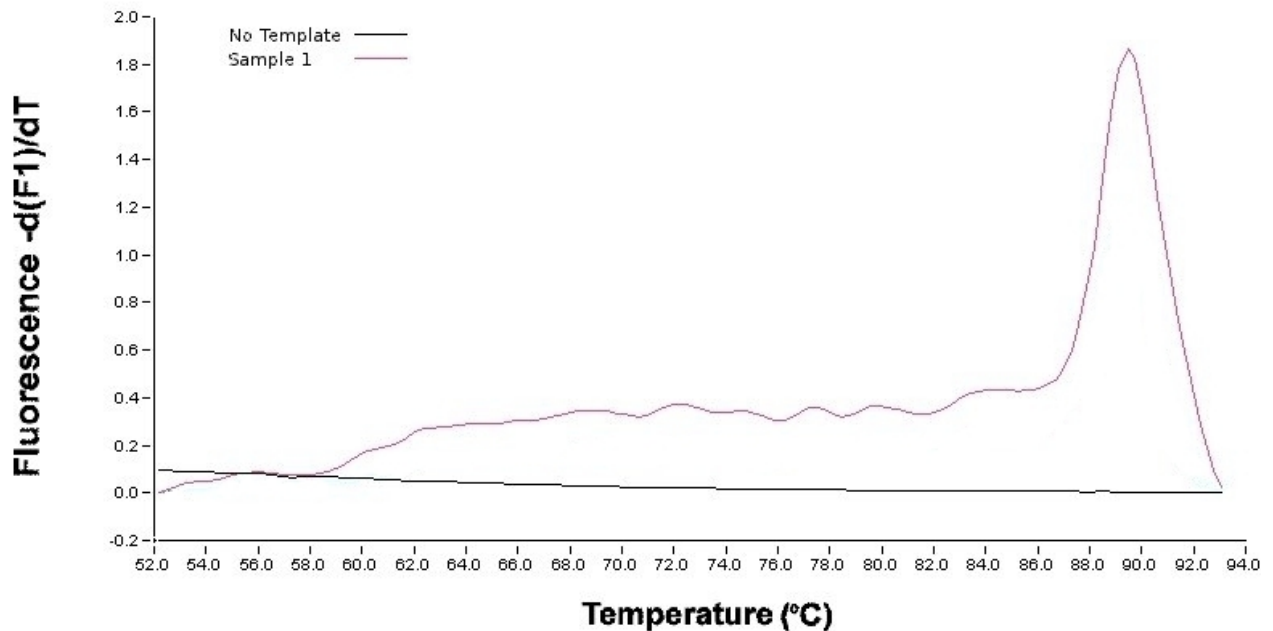

Figure C3: Representation of a melting peak curve for MIP-1β amplification products. The black line shows the curve of a sample containing no template and serves as a negative control. The purple line represents the melting peak of mRNA isolations from Vk2/E6E7 cells that were exposed to 0.01% (v/v) EtOH and 0.02 μg/ml TNFα for 2h.

ADDENDUM D: BUFFERS AND SOLUTIONS

Passive Lysis Buffer
0.5 ml Triton X-100
25 ml Glycerol
7 ml Tris-phosphate-EDTA
720 μl 0.5 M EDTA
Final volume to 250 ml using deionised water
Store at 4ºC

5X Laemmli Buffer
1 ml 1 M Tris-HCl buffer pH 6.8
5 ml 10% (w/v) SDS
0.01 g Bromophenol Blue
2 ml glycerol
0.5 ml Beta-Mecapto-EtOH
Final volume to 10 ml using deionised water
Store at -20ºC

10% (w/v) SDS (pH 7.2)
100g SDS
Dissolve at 68˚C and adjust pH to 7.2 with HCl
Final volume to 1 L using reverse osmosis (RO) water
Store at room temperature

1 M Tris-HCl Buffer (pH 6.8)
121.4 g tris(hydroxymethyl)aminomethane
Adjust pH to 6.8 with HCl
Final volume to 1 L using RO water
Sterilize by autoclaving and store at room temperature.

1.5 M Tris-HCl Buffer (pH 8.8)
182.1 g tris(hydroxymethyl)aminomethane
Adjust pH to 8.8 with HCl
Final volume to 1 L using RO water
Sterilize by autoclaving and store at room temperature.

10% (w/v) adenosine 5'-phosphosulphate
1g adenosine 5'-phosphosulphate
Final volume to 10 ml using deionised water
Store at -20˚C

30% (w/v) acryl-bisacrylamide
29 g acrylamide
1g N, N'-methylenebisacrylamide
Dissolve at 37˚C in RO water
Final volume to 100 ml using RO water
Store at 4˚C protected from light

10X SDS PAGE Running buffer (pH 8.3)
10 g SDS
30.3 g Tris
144.1 g glycine
Final volume to 1 L using RO water
Store at room temperature.

1X Transfer buffer
6.1 g Tris
28.8 g glycine
200 ml Methanol
Final volume to 1 L using RO water
Store at 4˚C

1X TBS (Tris Buffered Saline)
6.05 g Tris
8.76 g NaCl
Adjust pH to 7.5 with HCl
Final volume to 1 L using reverse osmosis water
Store at 4ºC

1X TBST (Tris Buffered Saline - 0.1% Tween)
1 ml Tween 20
1 L TBS buffer
Store at 4ºC

LB medium
10 g NaCl
10 g Tryptone
5 g yeast extract
Final volume to 1 L using deionised water
Sterilize by autoclaving and store at room temperature.

Ampicillin stock (50 mg/ml)
0.75 g ampicillin
Final volume to 25 ml using deionised water
Filter sterilize using a 0.22 micron filter
Store at -20°C

LB Agar Plates
2.5g tryptone
2.5g NaCl
1.25g yeast extract
3.75g bacterial agar
Final volume to 250 ml using deionised water
Autoclave and cool
Add ampicillin to a final concentration of 50 μg/ml

0.5M EDTA
93.06g EDTA
Adjust pH to 8.2 with NaOH
Final volume to 500ml with RO water
Store at room temperature

ADDENDUM E: VPR EFFECTS ON MIP-1β EXPRESSION

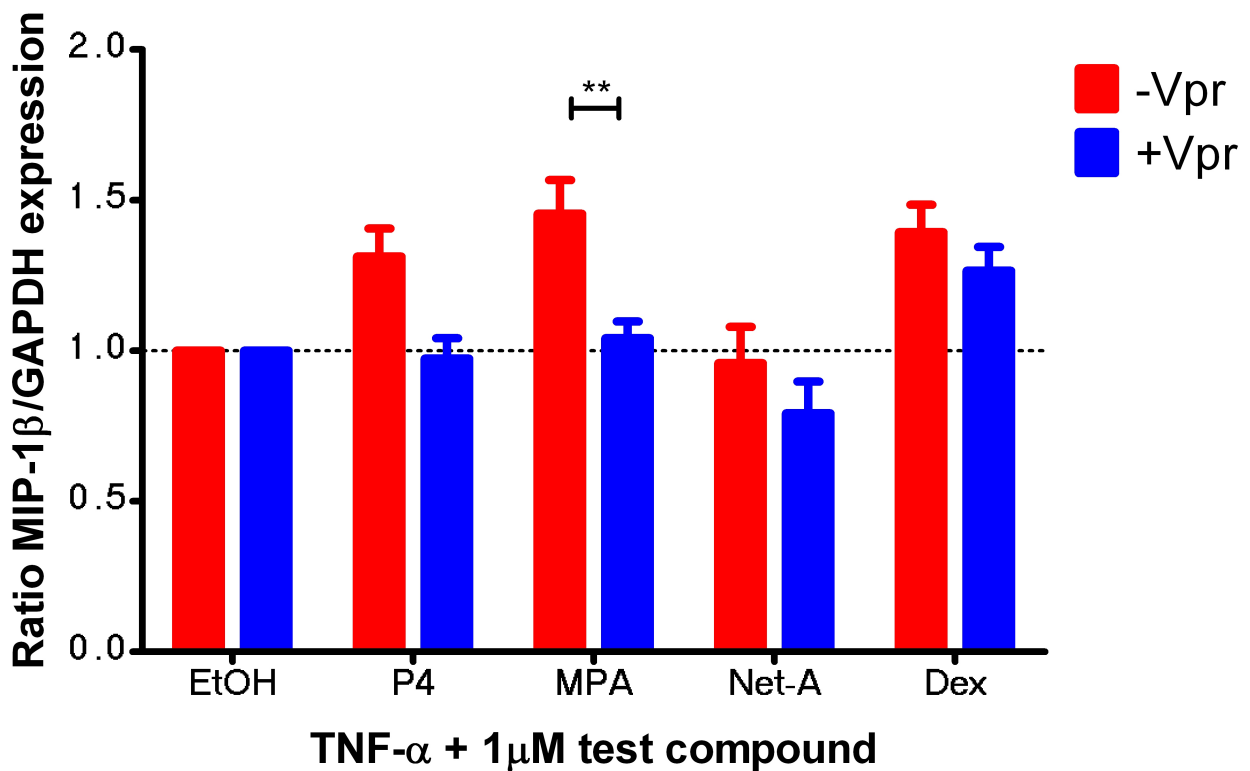*Vpr modulates ligand induced expression of MIP-1β in the Vk2/E6E7 cell line.*

Figure E1: The effect of Vpr on P4, MPA and Dex induced mRNA expression of MIP-1β in the Vk2/E6E7 cell line. Vk2/E6E7 cells were transfected with 1000 ng pCMV4-3HA·Vpr. Twenty-four hours later, cells were incubated with 0.02 μg/ml TNF-α and 1μM P4, MPA, Net-A or Dex for 2h. Total RNA was isolated, cDNA synthesized, and analysed using QPCR with primers specific to MIP-β and the internal control, GAPDH. Relative expression of MIP-1β was normalised to relative GAPDH expression. For statistical analysis two-way ANOVA was used with Bonferroni as post test (* p<0.05, ** p<0.01, and *** p<0.001). The result represents the average (±SEM) of at least two independent experiments.

ADDENDUM F: PUBLICATION

Co-author of publication Africander *et al.* (11) for determining the ER levels using whole cell binding.

Original research article

Differential regulation of endogenous pro-inflammatory cytokine genes by medroxyprogesterone acetate and norethisterone acetate in cell lines of the female genital tract

Donita Africander[a], Renate Louw[a], Nicolette Verhoog[b], Dewald Noeth[a], Janet P. Hapgood[b],*

[a]*Department of Biochemistry, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa*
[b]*Department of Molecular and Cell Biology, University of Cape Town, Private Bag X3, Rondebosch, 7701, Western Cape, South Africa*

Abstract

Background: Medroxyprogesterone acetate (MPA) and norethisterone (NET) and its derivatives are widely used in female reproductive therapy, but little is known about their mechanisms of action via steroid receptors in the female genital tract. MPA used as a contraceptive has been implicated in effects on local immune function. However, the relative effects of progesterone (Prog), MPA and norethisterone acetate (NET-A) on cytokine gene expression in the female genital tract are unknown.
Study Design: Using two epithelial cell lines generated from normal human vaginal (Vk2/E6E7) and ectocervical (Ect1/E6E7) cells as in vitro cell culture model systems for mucosal immunity of the female cervicovaginal environment, we investigated steroid receptor expression and activity as well as regulation of cytokine/chemokine genes by MPA and NET-A, as compared to the endogenous hormone Prog.
Results: We show that the Prog, androgen, glucocorticoid and estrogen receptors (PR, AR, GR and ER, respectively) are expressed in both the Vk2/E6E7 and Ect1/E6E7 cell lines, and that the GR and AR are transcriptionally active. This study is the first to show ligand-, promoter- and cell-specific regulation of IL-6, IL-8 and RANTES (regulated-upon-activation, normal T cell expressed and secreted) gene expression by Prog, MPA and NET-A in these cell lines. Moreover, we show that the repression of the TNF-α-induced RANTES gene by MPA in the Ect1/E6E7 cell line is mediated by the AR.
Conclusion: Collectively, these data demonstrate that cell lines from different anatomical sites of the female genital tract respond differently to Prog and the synthetic progestins, most likely due to differential actions via different steroid receptors. The results highlight the importance of choice of progestins for immune function in the cervicovaginal environment. They further suggest that choice of progestins in endocrine therapy may have implications for women's risk of susceptibility to infections due to differential actions on genes involved in inflammation and immune function.
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Keywords: Synthetic progestins; Injectable contraceptives; Female genital tract; Cytokines

1. Introduction

The mucosal surface of the lower female genital tract (cervicovaginal environment) is a complex system that provides a barrier against pathogens. Epithelial cells lining the cervicovaginal mucosa are the point of entry for many viral, bacterial and parasitic infections [1,2]. These cells thus have features enabling them to combat infections, such as the expression of cytokines, hormone receptors and genital tract-specific defensins [3–6] (and reviewed in Ref. [7]). Cervicovaginal epithelial cells constitutively express a wide variety of pro- and anti-inflammatory mediators, such as the cytokines, interleukin (IL)-1, IL-6, IL7, macrophage colony-stimulating factor, transforming growth factor beta and the chemoattractant cytokines, IL-8 and RANTES (regulated-upon-activation, normal T cell expressed and secreted), which are up-regulated in response to tumor necrosis factor (TNF)-α [1,2,8]. These cells thus play an important role in the innate and acquired immune systems present at the mucosal surfaces.

IL-8 and RANTES (also termed CCL5) are chemotactic cytokines, or chemokines, involved in the early inflammatory response by recruiting specific leukocytes, particularly macrophages, to sites of ongoing inflammation and injury, while IL-6 is responsible for neutrophil priming to chemotactic factors [9]. Interestingly, increased levels of the cytokines IL-

* Corresponding author.
E-mail address: janet.hapgood@uct.ac.za (J.P. Hapgood).

1, TNF-α, IL-6 and the chemokines RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β in cervicovaginal secretions have been associated with human immunodeficiency virus (HIV)-1 infection and bacterial vaginosis [10–12]. Moreover, decreased systemic CD4$^+$ cell counts during acute HIV infection has been associated with increased levels of IL-1β, IL-6 and IL-8 in genital tract secretions [13]. Thus, the cytokine milieu in the cervicovaginal mucosa is an important determinant of resistance and susceptibility to infections.

The transmission of and susceptibility to infections in women may be better understood if factors affecting the immune response in the vagina and cervix are more clearly defined. Research in animal models and in women indicates that local as well as regional immune responses affect the outcome of vaginal challenge with microbial pathogens (reviewed in Ref. [14]). Sex hormones are examples of factors that have been shown to influence susceptibility and disease predisposition to many genital tract infections [15]. Furthermore, there are indications that women using antibiotics, corticosteroids (immunosuppressive therapy), oral contraceptives and hormone replacement therapy (HRT) are more susceptible to fungal vaginal infections [16–18].

The synthetic progestins medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN) are the most widely used injectable female contraceptives, with at least 20 million current users of MPA worldwide [19]. MPA itself, rather than its metabolites, is the major progestogenic compound, while NET-EN and norethisterone acetate (NET-A) are hydrolyzed to norethisterone (NET) and its metabolites, which together have progestogenic action [20]. Both MPA and NET are also used for HRT in postmenopausal women. MPA used as contraceptive has been shown to increase HIV [21] and HSV cervical shedding in HIV-infected women [22]. Although Mostad et al. [21] did not investigate the molecular mechanism of these effects, they postulate that the effects may be mediated by factors such as direct effects on the virus, effects on local genital tract physiology or effects on immune modulation of viral replication, or a combination of these effects. Interestingly, an animal study showed that MPA increased susceptibility to vaginal simian–human immunodeficiency virus (SHIV) transmission and suppressed the antiviral cellular immune response in SHIV-infected rhesus macaques [23], indicating an immune- rather than a transmission-based mechanism. In another animal study, it was shown that MPA treatment at contraceptive doses rendered mice 100-fold more susceptible to genital HSV-2 infection compared to untreated mice [24]. In addition, the use of MPA has been associated with increased acquisition of cervical chlamydial and gonococcal infections [25]. Whether or not NET is associated with an increased risk of HIV/HSV acquisition and shedding, viral load and viral diversity remains to be determined. In this regard, two recent studies in a cohort of South African women showed no association between risk of HIV infection and NET-EN at contraceptive doses [26,27]. However, a recent re-analysis of earlier data has shown that MPA used as an injectable contraceptive (referred to in this case as Depo-MPA or DMPA) is associated with an increase in HIV acquisition in women [28,29].

To date, very little is known about the molecular mechanisms of action of MPA and NET on immune function, in particular the target cells, target genes and dose responses. MPA has been reported to modulate transcription of a number of genes via the glucocorticoid receptor (GR), for example, IL-2 in normal human lymphocytes [30], IL-6 and IL-8 in a mouse fibroblast cell line [31] and the *nm-23* tumor suppressor gene in a breast cancer cell line [32]. In contrast, MPA's suppression of the RANTES gene in endometrial cells was progesterone receptor (PR) mediated [33]. Interestingly, NET-A is not an agonist for transactivation via the GR and only marginally (22%) transrepressed an IL-8 reporter at 10 μM [34]. Furthermore, MPA has been shown to regulate a number of genes via the PR and the androgen receptor (AR) in human breast cancer cell lines [35]. In contrast to the data available for MPA, much less is known about the biological activity of NET via the AR. However, the recent study by Sasagawa et al. [36] has now characterized both MPA and NET pharmacologically in terms of potency for transactivation via the AR. Furthermore, both MPA and NET-A have been reported to bind to the mineralocorticoid receptor (MR) with low relative affinity and do not display any agonist or antagonist properties towards the MR [37,38]. It is thus apparent that even though MPA and NET were developed for the similarity of their biological actions to those of progesterone (Prog), mediating their effects by binding to the PR, they can also initiate a diverse range of biological effects by cross-reacting with other members of the steroid receptor family such as the GR, the AR and possibly the MR [34,39–42].

An important question is thus whether, and to what extent, MPA, and also NET-A, regulates known pro-inflammatory mediators such as IL-6, IL-8 and RANTES, in the cervicovaginal mucosa. Understanding the mechanisms of this regulation and the receptors involved would further our understanding of differential gene regulation by different progestins and assist in the design of new progestins with fewer side effects. Investigating these mechanisms at a site relevant to infections, such as the cervicovaginal environment, is likely to be relevant to mucosal immunity. Factors that affect immunity in the cervicovaginal environment may be important determinants of transmission risk of pathogens such viruses, and understanding these factors may shed light on molecular events occurring during infections. In the light of the above, our strategy was to investigate the effects of MPA and NET-A relative to Prog on mucosal immunity in an in vitro cell culture model of the female cervicovaginal environment, by comparing their effects on the regulation of the endogenous pro-inflammatory cytokine/chemokine genes IL-6, IL-8 and RANTES. Two epithelial cell lines generated from normal human vaginal (Vk2/E6E7) and ectocervical (Ect1/E6E7) cells, immortalized by expression of the E6 and E7 genes of human papillomavirus type 16 [42], were used as model systems.

2. Materials and methods

2.1. Inducing compounds

4-Pregnene-3,20-dione (Prog), 6α-methyl-17α-hydroxy-progesterone acetate (MPA), 17α-ethynyl-19-nortestoster-one 17β-acetate (NET-A), 5α-androstan-17β-ol-3-one [di-hydrotestosterone (DHT)], 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one [mifep-ristone (RU486)], 11β,16α-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione [dexamethasone (Dex)], 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al [al-dosterone (Ald)], 17β-estra-1,3,5 (10)-triene-3,17-diol [β-estradiol (E₂)] and recombinant TNF-α were obtained from Sigma-Aldrich (South Africa). NET-A, the acetate, was used as it is soluble in water compared with the insoluble ester of NET-EN. In vivo, both NET-EN and NET-A undergo hydrolysis and are converted to NET and its metabolites [20]. Hydroxyflutamide (OHF) was obtained from Dr. C. Tendler (Schering Plough Research Institute, USA). Unla-belled 17β-hydroxy-7α,17α-dimethylestr-4-en-3-one [mibolerone (MIB)], [³H]-MIB (76.8 Ci/mmol), unlabelled R5020, ³[H]-R5020 (84.6 Ci/mmol) and [³H]-Ald (87.9 Ci/mmol) were purchased from PerkinElmer Life and Analytical Science (South Africa). [³H]-Dex (89 Ci/mmol) and ³[H]-E₂ (110 Ci/mmol) were purchased from AEC-Amersham (South Africa). All unlabelled test compounds were dissolved in absolute ethanol (1 and 10 mM stock concentrations) and stored at −20°C. These compounds were then added to serum-free culturing medium such that the final concentra-tion of ethanol was 0.1%. Control incubations (no test compounds) were performed in the presence of 0.1% ethanol.

2.2. Cell culture

Ect1/E6E7 and Vk2/E6E7 cell lines (human ectocervical and vaginal epithelial cell lines, respectively) were purchased from American Type Culture Collection (ATCC, USA) and main-tained in keratinocyte serum-free medium (GibcoBRL, Paisley, UK) supplemented with 50 mcg/mL bovine pituitary extract (GibcoBRL), 0.1 ng/mL epidermal growth factor (GibcoBRL), 0.4 mM CaCl₂, 50 IU/mL penicillin and 50 mcg/mL streptomycin (GibcoBRL). These cells have been validated as a model for epithelial immune function by comparisons with primary cell cultures, tissues, animal models and clinical findings, and show similar toll-like receptor and cytokine profiles to primary cells [1,3,43]. Cultures were maintained in 75-cm² culture flasks (Greiner Bio-One International, Austria) at 37°C, in an atmosphere of 90% humidity and 5% CO₂. All cultures were regularly tested for mycoplasma infection by means of Hoechst staining [44], and only mycoplasma-negative cell lines were used in experiments.

2.3. Plasmids

Plasmids expressing the human mineralocorticoid receptor pRShMR and the human glucocorticoid receptor pRS-hGRα were a kind gift from Prof. R. Evans (Howard Hughes Medical Institute, La Jolla, CA, USA). A plasmid expressing the human androgen receptor pSVARo [45] was obtained from Frank Claessens, (University of Leuven, Leuven, Belgium). The plasmid expressing the human progesterone receptor (isoform B) pSG5hPR-B [46] was obtained from Stoney Simons, Jr. (NIH, Bethesda, USA), while the human estrogen receptor-α (pSG5-ERα) and -β (pSG5-ERβ) expression vectors were received from Prof. Frank Gannon, European Molecular Biology Laboratory (EMBL, Heidelberg, Germany). The pGL2basic empty vector and the pSV-β-galactosidase expression vector (pSV-β-gal) were obtained from Promega (Madison, WI, USA). The plasmid pTAT-GRE-E1b-luc, driven by the E1b promoter and containing two copies of the rat TAT-GRE was obtained from Dr. G. Jenster (Erasmus University of Rotterdam, The Netherlands). The 5xNFκB-luc plasmid and the cytomegalovirus (CMV)-driven-β-galactosi-dase expression vector (pCMV-β-gal) were from Stratagene (Houston, TX, USA).

2.4. Isolation of total RNA and real-time quantitative RT-PCR

Ect1/E6E7 and Vk2/E6E7 cell lines were maintained as described above. Cells were induced with 0.02 mcg/mL TNF-α and 1 μM of the test compounds, in the absence and presence of 10 μM receptor-specific antagonists, for 24 h. Total RNA was isolated from cells using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. The integrity of the RNA (presence of intact 18S and 28S ribosomal bands) was confirmed by denaturing agarose gel electrophoresis. Total RNA (1 mcg) was reverse transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, South Africa) according to the manufacturer's instructions. Real-time PCR was performed by using a LightCycler, rapid thermal cycler system (Roche Applied Science) according to the manufacturer's instruc-tions. Nucleotides, TaqDNA polymerase and buffer used in the reaction were those included in the LightCycler-FastStart DNA Master[PLUS] SYBR Green I system (Roche Diagnos-tics, South Africa). Agarose gel electrophoresis and melting curve analysis were performed to confirm the generated amplicon in each sample. The amplification efficiency for each primer was determined by generating a standard curve from a cDNA dilution series. The efficiencies were 1.99, 1.92, 1.98 and 2.0 for IL-6, IL-8, RANTES and glyceral-dehyde-3-phosphate dehydrogenase (GAPDH), respectively. Relative IL-6, IL-8 and RANTES transcript levels were calculated with the Fit Points method described by Pfaffl [47] and were normalized to relative GAPDH transcript levels. The specific primers used were as follows: for RANTES, 5′-TACCATGAAGGTCTCCGC-3′ (forward primer) and 5′-GACAAAGACGACTGCTGG-3′ (reverse primer) [48]; for IL-6, 5′-TCTCCACAAGCGCCTTCG-3′ (forward primer) and 5′-CTCAGGGCTGAGATGCCG-3′ (reverse primer) [48]; for IL-8, 5′-TGCCAAGGAGTGC-TAAAG-3′ (forward primer) and 5′-CTCCACAA-CCCTCTGCAC-3′ (reverse primer) [48]; for GAPDH,

5′- TGAACGGGAAGCTCACTGG-3′ (forward primer) and 5′- TCCACCACCCTGTTGCTGTA-3′ (reverse primer [49]. The product sizes were 199, 193, 197 and 307 bp for RANTES, IL-6, IL-8 and GAPDH, respectively.

2.5. Western blotting

Ect1/E6E7 and Vk2/E6E7 cell lines were plated at 1×10^6 cells per well in six-well plates and allowed to grow to confluency. Cells were washed with phosphate-buffered saline (PBS) and lysed in 100 μL SDS-sample buffer [50]. Lysates were boiled and subjected to electrophoresis on an 8% SDS polyacrylamide gel using β-actin expression as a loading control. Following electrophoresis, proteins were transferred to nitrocellulose membranes. Blots were probed with anti-AR (H-280, 1:1000), anti-GR (H300, 1:3000), anti-PR-B (sc-811, 1:500), anti-PR-AB (sc-810, 1:500), anti-MR (H300, 1:1000), anti-ER-α (MC-20, 1:200) or anti-ER-β (H-150, 1:1000) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA), as well as the anti-β-actin (1:1500) antibody from Cell Signalling Technology (Massachusetts, USA), for 16 h at 4°C. Blots were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:10,000 for GR and β-actin, 1:2000 for MR, 1:5000 for ER-α, 1:1000 for ER-β; NA934VS, Amersham, South Africa) or goat anti-mouse secondary antibody (1:5000 for AR, 1:2000 for PR-A and PR-B; Santa Cruz Biotechnology) for 1 h. Proteins were visualized using enhanced chemiluminescence from Amersham, followed by exposure to Hyperfilm MP high-performance autoradiography film (Amersham, South Africa).

2.6. Whole cell binding assays

Competitive whole cell binding assays were performed essentially as described by Bamberger et al. [51], with the following modifications. Ect1/E6E7 and Vk2/E6E7 cell lines were maintained as described above. Cells were seeded into 12-well tissue culture plates (Nunc, Denmark) at 2×10^5 cells per well. On Day 3, the cells were washed with PBS and incubated for 2 h at 37°C with 10 nM [^{3}H]-MIB (76.8 Ci/mmol) for AR, [^{3}H]-Ald (87.9 Ci/mmol) for MR, [^{3}H]-R5020 (84.6 Ci/mmol) for PR (^{3}[H]-MIB, ^{3}[H]-Ald and ^{3}[H]-R5020 all from PerkinElmer Life and Analytical Science, South Africa), ^{3}[H]-Dex (89 Ci/mmol) for GR (AEC-Amersham) or ^{3}[H]-E$_2$ (110 Ci/mmol) for ER (AEC-Amersham), in the absence (total binding) or presence of 10 μM unlabelled MIB, Ald, R5020, Dex or E$_2$ (nonspecific binding), respectively. Working on ice at 4°C, cells were washed three times with ice-cold 1× PBS containing 0.2% (w/v) bovine serum albumin for 15 min. Cells were then lysed with a 200 μL reporter lysis buffer (Promega). Total binding was determined by scintillation counting as counts per minute (cpm), while specific binding was determined by subtracting nonspecific binding from total binding. Protein concentrations were determined using the Bradford protein assay method [52]. Specific binding was normalized to the protein concentration. The reported values are averages of at least three independent assays, with each condition performed in triplicate. The receptor numbers in femtomole per milligram of protein were determined as follows.

The specific activity of the tritium-labelled test compound (Ci/mmol) was converted to disintegrations per minute (dpm) per millimole, by multiplying by a factor of 2.22×10^{12} (1 Ci equals 2.22×10^{12} dpm). The disintegration per minute was multiplied by the counting efficiency to get the counts per minute per millimole and divided by 10^{12} to get the counts per minute per femtomole. Counting efficiency was 40% in our system. The specific activity was expressed as counts per minute per femtomole. Dividing the specific binding counts per minute value by this value gives specific binding in femtomoles, which was divided by the protein content of the sample in milligrams, to yield the number of binding sites in femtomoles per milligram of protein.

2.7. Luciferase reporter assays

For transactivation assays, cells were maintained as above and seeded into 24-well tissue culture plates at 5×10^4 cells per well. On Day 2, cells in each well were transiently transfected with 330 ng of the pTAT-GRE-E1b-luc construct and 33 ng of the expression vector for the hGR, hAR, hPR-B, hMR or the empty vector pGL2basic, using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals, South Africa) in accordance with the manufacturer's instructions. On Day 3, the cells were washed with PBS and induced with vehicle (0.1% EtOH) or 10 μM of either Dex, MIB, R5020 or Ald. For transrepression assays, cells were maintained as above and seeded into 24-well tissue culture plates at 5×10^4 cells per well. On Day 2, the cells were transiently transfected with (1) 100 ng of the 5xNF-κB-luc construct, either 50 ng of the expression vector for the hGR or the empty vector pGL2-basic, and 25 ng pSV-βgal or with (2) 400 ng of the 5xNF-κB-luc construct, either 70 ng of the expression vector for the hAR or pGL2-basic, and 70 ng pCMV-βgal, using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. On Day 3, the cells were stimulated with 0.02 mcg/mL TNF-α in the absence (0.1% ethanol) and presence of 10 μM Dex (for the GR) or MIB (for the AR). After 24 h, the cells were lysed with reporter lysis buffer (Promega). Luciferase activity in the lysate was measured using the Luciferase Assay System (Promega) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). The values obtained were normalized to expression of β-gal, which was measured using the GalactoStar Assay Kit from Tropix (Bedford, MA, USA). The reported values are averages of at least three independent experiments, with each condition performed in triplicate.

2.8. Data manipulation and statistical analysis

The GraphPad Prism software was used for data manipulations, graphical representations and statistical analysis. One-way ANOVA analysis of variance and Dunnett (compare all columns vs. control column) or Bonferroni post-

tests (compares all pairs of columns) were used for statistical analysis. In some figures, statistical significance of differences is indicated by *, ** or ***, to indicate $p<.05$, $p<.01$ or $p<.001$, respectively, whereas no statistical significance ($p>.05$) is indicated by 'ns'. In other figures, the letters 'a', 'b', 'c', etc., are also used to denote statistically significant differences, where all those values which differ significantly from others are assigned a different letter.

3. Results

3.1. MPA and NET-A, unlike Prog, exhibit differential patterns of gene regulation on pro-inflammatory chemokines

Human Ect1/E6E7 and Vk2/E6E7 cell lines were treated for 24 h with 0.02 mcg/mL TNF-α and 1 µM Prog, MPA or NET-A, followed by real-time quantitative RT-PCR (QPCR) analysis for expression of the IL-6, IL-8 and RANTES genes, respectively. In both the Ect1/E6E7 and Vk2/E6E7 cell lines, results show that unlike Prog which up-regulates IL-6 gene expression, both MPA and NET-A have no effect (Fig. 1A and 1B). In contrast, MPA and NET-A differentially regulate both the IL-8 (Fig. 2A) and RANTES (Fig. 3A) genes in the Ect1/E6E7 cell line. The results show that MPA, like Prog, significantly up-regulates IL-8 (10.3- vs. 12-fold, respectively), while NET-A has no significant effect (Fig. 2A). Although not statistically significantly different, Prog and NET-A seemed to up-regulate the IL-8 gene in the Vk2/E6E7 cell line to a similar extent, while MPA appeared to show the greatest level of up-regulation (Fig. 2B). Unlike Prog which significantly up-regulates RANTES gene expression in the Ect1/E6E7 cell line, MPA significantly down-regulates RANTES gene expression, while NET-A has no effect (Fig. 3A). Conversely, both MPA and NET-A, like Prog, appeared to slightly up-regulate the expression of the RANTES gene in the Vk2/E6E7 cell line (Fig. 3B). Taken together, MPA and NET-A exhibit differential patterns of gene regulation on expression of the IL-8 and RANTES genes, as compared to Prog, in a promoter- and cell-specific manner. In addition, unlike Prog, MPA and NET-A do not up-regulate IL-6 gene expression.

3.2. The PR, AR, GR and ER are expressed in both ectocervical and vaginal cell lines

As these progestins are known to interact with the PR, AR, GR and possibly the MR [36], we hypothesized that the differential regulation of genes by MPA and NET-A, particularly on the RANTES gene, was due to their action via different steroid receptors or to differing activities via the same steroid receptor. To test this hypothesis, we firstly investigated which members of the steroid receptor family were expressed in these cell lines using a combination of Western blotting (Fig. 4A) and whole cell binding (Fig. 4B and C).

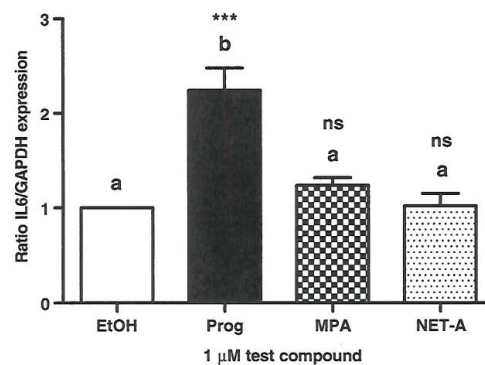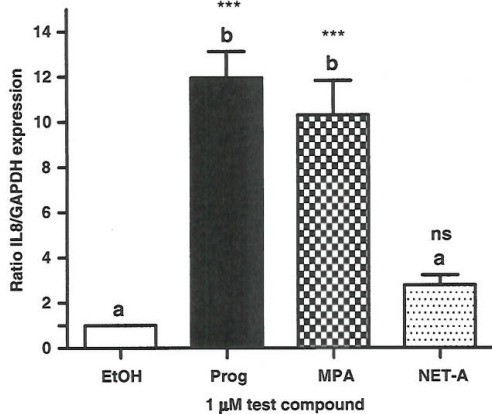**A ECTOCERVICAL CELLS**
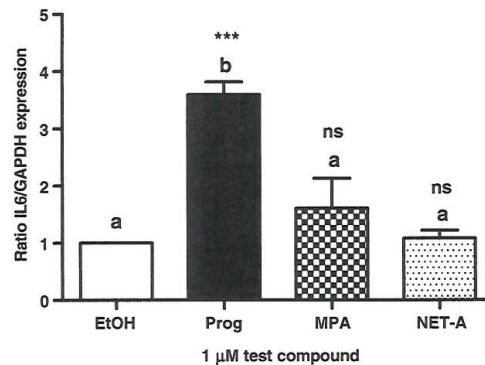
B VAGINAL CELLS

Fig. 1. Effect of MPA and NET-A on the TNF-α-induced expression of the IL-6 gene in human ectocervical and vaginal epithelial cell lines. (A) Human Ect1/E6E7 and (B) Vk2/E6E7 cell lines were incubated with 0.02 mcg/mL TNF-α in the presence of 0.1% EtOH or 1 µM Prog, MPA or NET-A for 24 h. Total RNA was extracted, cDNA synthesized and expression levels of IL-6 and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least three independent experiments. One-way ANOVA and both Bonferroni (compares all pairs of columns) and Dunnett [compares all columns vs. control (EtOH) column] post-tests were used for statistical analysis. The letters 'a', 'b', 'c', etc., are used to denote statistically significant differences, where all those values which differ significantly from others are assigned a different letter. Statistical significance of differences indicated by *, ** or *** indicates $p<.05$, $p<.01$ or $p<.001$, respectively, whereas no statistical significance ($p>.05$) is indicated by 'ns'.

Probing with an antibody specific for the PR-B isoform in the Western blot indicated that neither of these cell types expresses PR-B. We were unable to optimize the commercial antibody detecting the PR-A isoform [anti-PR-AB (sc-810), Santa Cruz Biotechnology] and thus could not determine by Western blotting whether the PR-A isoform is expressed in these cell lines. Similar levels of the AR and GR were detected in both cell lines by Western blotting (Fig. 4A).

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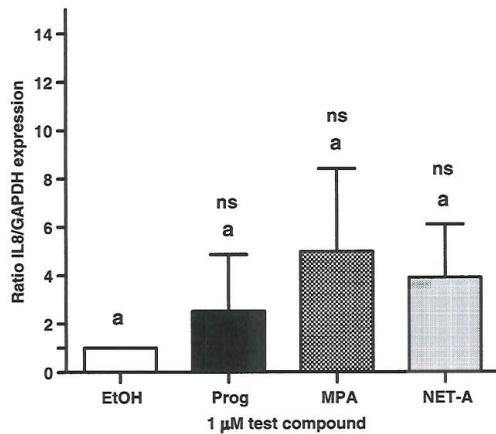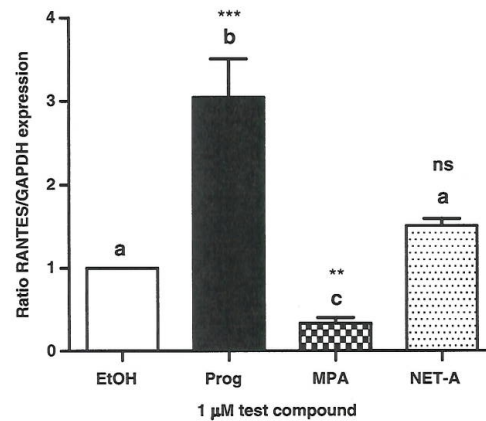
A ECTOCERVICAL CELLS

B VAGINAL CELLS

Fig. 2. Effect of MPA and NET-A on the TNF-α-induced expression of the IL-8 gene in human ectocervical and vaginal epithelial cell lines. (A) Human Ect1/E6E7 and (B) Vk2/E6E7 cell lines were incubated with 0.02 mcg/mL TNF-α in the presence of 0.1% EtOH or 1 μM Prog, MPA or NET-A for 24 h. Total RNA was extracted, cDNA synthesized and expression levels of IL-8 and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages ($\pm$SEM) of at least three independent experiments. One-way ANOVA and both Bonferroni (compares all pairs of columns) and Dunnett [compares all columns vs. control (EtOH) column] post-tests were used for statistical analysis. The letters 'a', 'b', 'c', etc., are used to denote statistically significant differences, where all those values which differ significantly from others are assigned a different letter. Statistical significance of differences indicated by *, ** or *** indicates p<.05, p<.01 or p<.001, respectively, whereas no statistical significance (p>.05) is indicated by 'ns'.

Interestingly, we also detected low levels of endogenous GR in the untransfected COS-1 cell line, consistent with its ubiquitous expression (reviewed in Ref. [53]). We were

unable to detect the presence of the MR by Western blotting in either the Ect1/E6E7 or the Vk2/E6E7 cell line, although the positive control indicated that the anti-MR antibody was
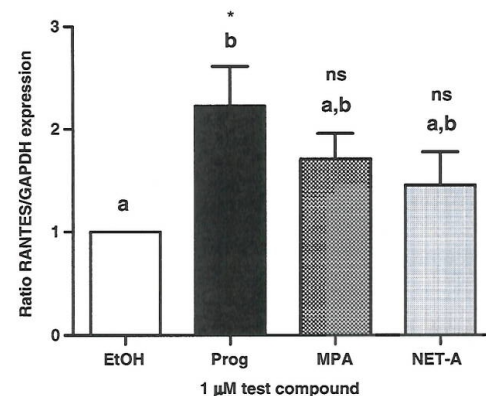
A ECTOCERVICAL CELLS

B VAGINAL CELLS

Fig. 3. Effect of MPA and NET-A on the TNF-α-induced expression of the RANTES gene in human ectocervical and vaginal epithelial cell lines. (A) Human Ect1/E6E7 and (B) Vk2/E6E7 cell lines were incubated with 0.02 mcg/mL TNF-α in the presence of 0.1% EtOH or 1 μM Prog, MPA or NET-A for 24 h. Total RNA was extracted, cDNA synthesized and expression levels of RANTES and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages ($\pm$SEM) of at least three independent experiments. One-way ANOVA and both Bonferroni (compares all pairs of columns) and Dunnett [compares all columns vs. control (EtOH) column] post-tests were used for statistical analysis. The letters 'a', 'b', 'c', etc., are used to denote statistically significant differences, where all those values which differ significantly from others are assigned a different letter. Statistical significance of differences indicated by *, ** or *** indicates p<.05, p<.01 or p<.001, respectively, whereas no statistical significance (p>.05) is indicated by 'ns'.
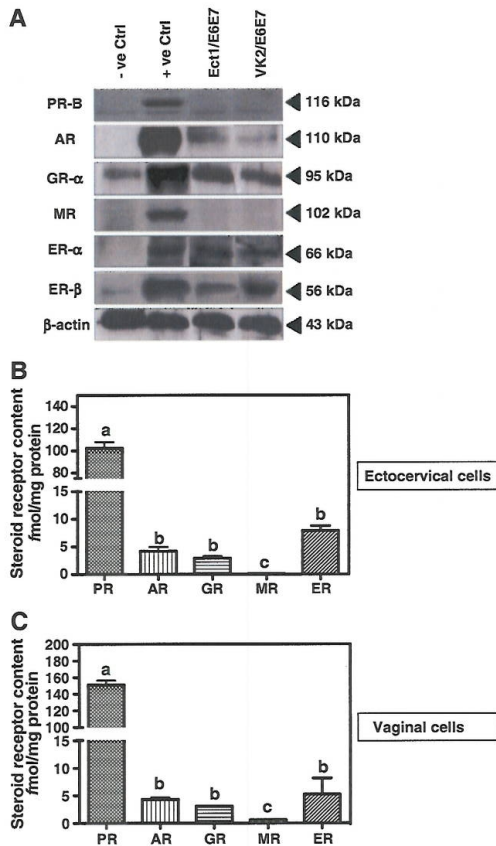
Fig. 4. PR, AR, GR and ER are expressed in human ectocervical and vaginal epithelial cell lines. (A) Whole cell extracts were prepared from human Ect1/E6E7 and Vk2/E6E7 cell lines; untransfected COS-1 cell line [negative control (−ve ctl)]; COS-1 cell line transfected with pSG5hPR-B, pSVARα, pRS-hGRα, pRS-hMR, pSG5-hERα or pSG5-hERβ expression vectors [positive controls (+ve ctl)]. Equal amounts of protein (20 mcg) were analyzed by Western blotting with the PR-B or AR or GR or MR or ERα or ERβ antibodies. (B) Human Ect1/E6E7 and (C) Vk2/E6E7 cell lines were incubated with 10 nM [^{3}H]-R5020 or [^{3}H]-MIB or [^{3}H]-Dex or [^{3}H]-Ald or [^{3}H]-E$_2$ in the absence (total binding) and presence of 10 μM unlabelled (nonspecific binding) R5020 or MIB or Dex or Ald or E$_2$, respectively. Specific binding (total binding minus nonspecific binding) is plotted. Results shown are the averages (±SEM) of at least three independent experiments with each condition performed in triplicate. The binding experiment for each receptor with its cognate ligand was performed independent of each other. Statistical significance was determined using a t test with a confidence level of .05, and the letters 'a', 'b', 'c', etc., are used to denote statistically significant differences (p<.05), where all those values which differ significantly from others are assigned a different letter. Statistical analysis showed the following results: PR vs. AR or GR or MR or ER (p<.001); MR vs. AR or GR or ER (p<.05); AR vs. GR vs. MR vs. ER (p>.05). The number of binding sites in femtomoles per milligram of protein (fmol/mg protein) was calculated from the specific activity of the radiolabelled ligands and from the specific binding counts per minute value, as described in the Materials and Methods section.

functional (Fig. 4A). Western blot analysis using antibodies specific for ER-α and ER-β indicated the presence of both isoforms in both cell lines.

Whole cell binding results with tritiated receptor-specific agonists confirmed that endogenous PR, AR, GR and ER are all expressed in both these cell lines (Fig. 4B and C). The results show that there is significantly (p<.001) more PR than AR, GR or ER in the Ect1/E6E7 and Vk2/E6E7 cell lines, respectively. Moreover, the levels of AR vs. GR vs. ER are similar to each other in both cell lines (p>.05), (Fig. 4B and C). These binding results are consistent with the detection of the GR, AR and ER in both cell lines by Western blotting (Fig. 4A). In contrast, the detection of relatively high amounts of PR in the binding assay appears to contradict the Western blot results. However, it is possible that the cells express the PR-A but not the PR-B isoform, and that the PR-A isoform is responsible for the binding activity observed in Fig. 4B and C. Although we did not detect any MR in these cells lines using Western blotting, the more sensitive whole cell binding assay indicated the expression of relatively very
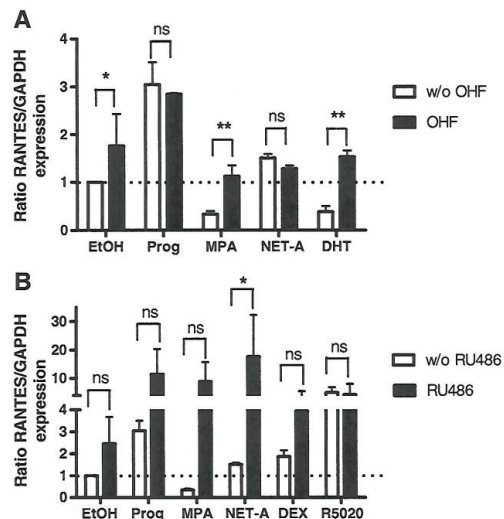

Fig. 5. Effect of the androgen receptor antagonist hydroxyflutamide (OHF) (A) or glucocorticoid/progesterone receptor antagonist (RU486) (B) on the MPA inhibition of the TNF-α-induced RANTES gene. The human Ect1/E6E7 cell line was incubated with 0.02 mcg/mL TNF-α in the presence of 1 μM Prog, MPA, NET-A, DHT, DEX or R5020, and absence or presence of 10 μM (A) OHF or (B) RU486 for 24 h. Total RNA was extracted, cDNA was synthesized and expression levels of RANTES and GAPDH mRNA were determined by QPCR. Results shown (ratio of the specific target gene/GAPDH gene) are the averages (±SEM) of at least two independent experiments. Open bars are compounds and vehicle only, while the black bars are compounds plus antagonist. Two-way ANOVA and Bonferroni post-test (compares replicate means by row) were used for statistical analysis. Statistical significance of differences is indicated by *, ** or *** to indicate p<.05, p<.01 or p<.001, respectively, whereas no statistical significance (p>.05) is indicated by 'ns'.

low amounts of MR in the Vk2/E6E7 cell line (Fig. 4C). Taken together, the results show that the expression of the PR, AR, GR and ER in both the Ect1/E6E7 and Vk2/E6E7 cell lines is consistent with our hypothesis that the differential effects of MPA and NET-A could be a consequence of their action via different steroid receptors.

3.3. Receptor-specific antagonists indicate a role for the AR in the down-regulation of the RANTES pro-inflammatory chemokine gene by MPA in the ectocervical cell line

Having determined that these cell lines express PR, AR, GR and ER, our strategy was to determine which of these steroid receptors mediates the differential effects of MPA and NET-A on the RANTES genes in the Ect1/E6E7 cell line. Although differential effects were also observed for MPA and NET-A on the expression of the IL-8 gene in the Ect1/E6E7 cell line, it was decided to continue the investigation only for the RANTES gene, as MPA and NET-A exhibited differential effects on this gene, as compared not only to each other, but also to Prog. For this reason, Ect1/E6E7 cells were incubated with 0.02 mcg/mL TNF-α and 1 μM Prog, MPA or NET-A, in the absence or presence of 10 μM OHF, the classical androgen antagonist, or RU486, the PR and GR antagonist. As most of the

literature indicates that MPA and NET-A do not bind to the ER [54–56], we did not investigate effects in the presence of an ER antagonist.

The addition of OHF prevented the inhibition by MPA, indicating that, in this cell line, the repression by MPA on the RANTES gene is mediated by the AR (Fig. 5A). Consistent with this result, DHT, the natural androgen, also repressed the RANTES gene in this cell line via the AR (Fig. 5A). Interestingly, the addition of OHF did not significantly change the response to Prog or NET-A, indicating that the effects of these compounds on the RANTES gene are not mediated via the AR. A similar experiment with OHF in the Vk2/E6E7 cell line indicated that the AR is not involved in any of the responses by Prog, MPA or NET-A on the RANTES gene (Supplementary Fig. S1).

However, in this cell line, RU486 did not inhibit the effects mediated by the positive controls — dexamethasone (Dex), a synthetic GR agonist, or promegestone (R5020), a synthetic PR agonist (Fig. 5B) — indicating that RU486 does not function as a GR or PR antagonist in the Ect1/E6E7 cell line, but rather as an agonist. This is consistent with previous results showing that RU486 may act as a GR agonist in some cells and as an antagonist in others [57]. The fact that the expression of the RANTES gene is enhanced when RU486 is added together with Prog, MPA or NET-A is
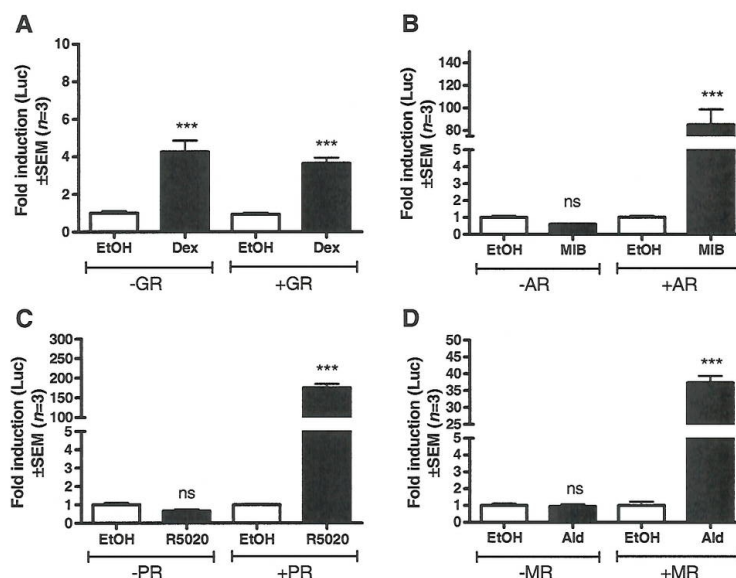

Fig. 6. Transactivation of a transiently transfected GRE-containing promoter–reporter construct via endogenous or expressed GR, AR, PR or MR. Ect1/E6E7 cells (5×10^4 cells per well in a 24-well plate) were transiently transfected with 330 ng pTAT-GRE2-Elb-luc and 33 ng pRS-hGRα, pSVARo, pSG5hPR-B, pRS-hMR or pGL2basic (empty vector control) as indicated, with FuGENE 6 transfection reagent according to the manufacturer's instructions. Twenty-four hours after transfection, cells were induced for 24 h with vehicle (EtOH), 10 μM Dex, MIB, R5020 or Ald (receptor agonists). One-way ANOVA and Dunnett post-test [compares all columns vs. control column (EtOH)] were used for statistical analysis. Statistical significance of differences is indicated by *, ** or *** to indicate p<.05, p<.01 or p<.001, respectively, whereas no statistical significance (p>.05) is indicated by 'ns'.

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difficult to interpret, but may indicate additive or synergistic effects. Taken together, conclusions cannot be drawn as to the role of the GR or PR in mediating the effects of any of these ligands in this cell line. Using a PR antagonist, like Org31710, which has little anti-glucocorticoid activity compared to RU486 [58], or a GR-specific antagonist like DO6 [59], was not possible since these antagonists are not commercially available. Nevertheless, the results with the

AR-specific antagonist showing a complete abrogation of the MPA response on the RANTES gene in the Ect1/E6E7 cell line suggest that the GR, PR, ER and MR are not involved in this MPA response.

3.4. Evidence for steroid receptor functional activity in promoter-reporter assays in both ectocervical and vaginal cell lines

The antagonist results showing that the effects of MPA on RANTES gene expression appear to be mediated predominantly via the AR are surprising given the apparent high PR expression levels based on binding activity, as well as expression of the GR. Both the PR and GR have been shown to have agonist or partial agonist activity, respectively, when bound to MPA [30,34]. We thus investigated by reporter assays whether the AR, PR, GR and MR are transcriptionally active by transiently transfecting the Ect1/E6E7 and Vk2/E6E7 cells with a steroid response element (SRE)-driven reporter construct containing two copies of the rat tyrosine amino transferase (TAT) GRE. The cells were subsequently exposed to 10 μM of the receptor-specific agonist Dex for the GR, MIB for the AR, R5020 for the PR and Ald for the MR. The results show that only the endogenous GR is transcriptionally active on an SRE reporter gene in the Ect1/E6E7 (Fig. 6) and Vk2/E6E7 (Supplementary Fig. S2) cell lines. These results with transfected receptors also show that the agonists used are active in these cell lines. However, results from transrepression experiments via a synthetic NFκB-promoter reporter indicate the presence of transcriptionally active GR and AR in the ectocervical cells (Fig. 7A and B). The detection of this transrepressive activity in the absence of transactivation activity is consistent with previous reports showing that the potency of a ligand for transactivation by the GR via a GRE is less than the potency for transrepression via NFκB [60]. These results suggest that the endogenous AR-mediated transrepressive activity by MPA on the RANTES gene may be mediated via NFκB sites.
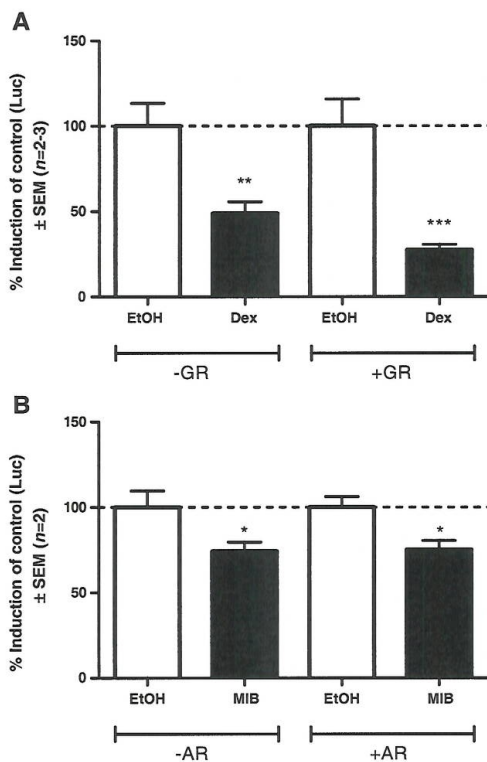

Fig. 7. Transrepression of a transiently transfected NFκB-containing promoter–reporter construct via endogenous or expressed GR or AR. Ect1/E6E7 cells (5×10^4 cells per well in a 24-well plate) were transiently transfected with (A) 100 ng NF-κB-luc, 50 ng pRS-hGRα or pGL2-basic and 25 ng pSV-βgal or with (B) 400 ng NF-κB-luc, 70 ng pSVARo or pGL2-basic and 70 ng pCMV-βgal, with FuGENE 6 transfection reagent according to the manufacturer's instructions. Twenty-four hours after transfection, cells were induced for 24 h with vehicle (0.1% EtOH) or 10 μM Dex or MIB (receptor agonists) and stimulated with 0.02 mcg/mL TNF-α. Percent induction by Dex or MIB, calculated as a percentage of EtOH+TNF-α (set as 100%), is plotted and indicated with dashed lines. Percent (%) repression was calculated by subtracting the induction in the presence of Dex or MIB from induction in the absence of ligand (EtOH=100%). One-way ANOVA and Dunnett post-test [compares all columns vs. control column (EtOH)] were used for statistical analysis. Statistical significance of differences is indicated by *, ** or *** to indicate p<.05, p<.01 or p<.001, respectively, whereas no statistical significance (p>.05) is indicated by 'ns'.

4. Discussion

Inflammation of the lower human female genital tract increases susceptibility to viral infections such as HIV [61,62] and human papilloma virus [63]. In addition, excessive release of proinflammatory cytokines may alter the mucosal immune function [64,65]. Thus, understanding factors that may influence the local mucosal immune response, such as endogenous hormones or hormonal contraception, is crucial, especially since the cervicovaginal mucosa is the primary site of HIV-1 infection during male-to-female transmission (reviewed in Ref. [66]). Although MPA and NET-A are widely used in both contraception and HRT, little is known about their effects on the cervicovaginal environment, and whether they interfere with local epithelial immune function. In this study, we investigated the effects of MPA, NET-A and Prog in two immortalized,

morphologically and functionally different epithelial cell lines, one from a normal human ectocervix and the other from a normal human vagina [42]. Although immortalized, the authors report that the morphological and immunocytochemical properties of these cell lines resemble not only their tissues of origin, but also those of primary cultures [42]. Notably, this is not the case for the HeLa cervical cell line, which is probably the most frequently used cell line derived from the human female lower genital tract mucosa [42]. The Ect1/E6E7 and Vk2/E6E7 cell lines express characteristics of stratified squamous nonkeratinizing epithelia and constitutively express IL-8, while IL-6 and RANTES are produced when the cell lines are stimulated with TNF-α [1].

The general trend that was observed for the effect of Prog, MPA and NET-A on endogenous cytokine genes in the cervicovaginal environment is that Prog up-regulates the expression of IL-6, IL-8 and RANTES in both the Ect1/E6E7 and Vk2/E6E7 cell lines, while NET-A either up-regulates these genes (Vk2/E6E7 cells) or has no effect (both cell lines). In contrast, MPA either has no effect (e.g., IL-6 gene in both cell lines) or up-regulates (e.g., IL-8 gene in both cell lines) or down-regulates (e.g., RANTES gene in Ect1/E6E7 cells) the expression of these genes. Interestingly, although MPA substantially and significantly down-regulates the expression of the pro-inflammatory RANTES gene in the Ect1/E6E7 cell line (Fig. 3A), it up-regulates the RANTES gene in the Vk2/E6E7 cell line (Fig. 3B). Thus, the same compound, MPA, exhibits differing effects on the same gene (RANTES), in different cell types, indicating cell-specific effects of MPA. In addition, NET-A has no statistically significant effect on the RANTES gene in both cell types, indicating differential regulation of RANTES by the two synthetic progestins thought to have similar biological activity. Furthermore, Prog shows regulation different to both MPA and NET-A on the RANTES gene in the Ect1/E6E7 cell line (Fig. 3A), as well as on the IL-6 gene in both the Ect1/E6E7 and Vk2/E6E7 cell lines (Fig. 1A and B). Taken together, these results show that cytokine genes in the cervicovaginal environment are regulated in a ligand-specific and cell-specific manner by Prog, MPA and NET-A.

The physiological implications of these results are potentially important. As IL-8 is a chemotactic factor for neutrophils and lymphocytes, the local concentration of IL-8 is important in determining recruitment of neutrophils and lymphocytes. Prog has previously been shown to induce a neutrophil response in the vagina of germ-free mice, even in the absence of bacterial infection [67], indicating a protective role for Prog in the vagina. Thus, our results showing that MPA and NET-A are similar to Prog in up-regulating IL-8, albeit to different extents, in the Ect1/E6E7 and Vk2/E6E7 cell lines, could indicate that these progestins may provide an enhanced local defense mechanism against infections in the vaginal mucosa. Interestingly, unlike Prog which up-regulates the expression of IL-6, MPA and NET-A had no effect on this gene. This may imply that the synthetic progestins would not be as protective as Prog in the cervicovaginal environment.

However, it may also indicate that the progestins would not lead to chronic inflammation in this environment, which could be positive in terms of susceptibility to pathogens such as HIV.

In epithelial cells, RANTES recruits immune cells into the reproductive tract, in this manner contributing to inflammation. Inhibition of RANTES by MPA would thus be expected to suppress inflammation, thereby blocking infiltration of immune cells [68]. This lack of recruitment of immune cells into the reproductive tract may have implications for women using MPA. In terms of HIV-1 infection, which has been associated with increased RANTES expression in cervicovaginal secretions [69], these implications may be positive in terms of less HIV infection due to decreased inflammation [64]. However, RANTES also acts as a natural HIV-1 entry inhibitor by competing with HIV-1 for the chemokine receptor CCR5 [70] (reviewed in Ref. [71]). In fact, RANTES peptide mimetics that display anti-HIV-1 activity are currently being developed as an HIV-1 entry inhibitor [72]. The suppression of RANTES by MPA may thus result in reduced competition with HIV-1 for CCR5, thereby allowing entry of HIV-1 into host cells and consequently leading to increased HIV-1 infection. In addition, women using MPA in contraception and HRT are also at risk of other infections, and thus the suppression of RANTES may prevent the mounting of an effective response to combat these infections in the ectocervical environment. Moreover, it is important to remember that the defense function in the cervicovaginal environment is dependent on number of regulatory factors in different anatomical sites, with a constant release of pro- and anti-inflammatory mediators. Thus, the observed effects of MPA on RANTES should be considered in the light of the fact that there may be a balance between pro- and anti-inflammatory molecules produced in the cervicovaginal environment. It would be of interest to investigate the expression of RANTES in cervicovaginal samples from healthy women and HIV-1-infected women using MPA and NET as injectable contraception, as compared to women not using these contraceptives.

We hypothesized that the ligand-specific and cell-specific effects on the regulation of cytokine genes in the cervicovaginal environment are due to ligands acting via different steroid receptors or to differing activities via the same steroid receptor. Using a combination of whole cell binding and Western blotting, we showed that the PR, AR, GR and ER are expressed in both cell lines (Fig. 4A–C). Whole cell binding experiments showed that the amount of GR, AR and ER in the Ect1/E6E7 and Vk2/E6E7 cell lines is similar (Fig. 4B and C). Since the whole cell binding experiments showed the presence of a large amount of PR, one would expect that the progestins would predominantly act via this receptor. However, we did not observe any PR-mediated effects in our study. In fact, the potent synthetic PR agonist, R5020, displayed no activity for transactivation via the PR (Fig. 6C and Supplementary Fig. S2(C)). A possible explanation for this intriguing result may be explained by the presence of different PR isoforms. We were unable to detect the PR-B isoform in any of the cell lines, and

thus we postulate that it is the transcriptionally less active PR-A isoform that is present in these cell lines. Indeed, variation in the expression of PR-A and PR-B has previously been reported in the eutopic endometrium during the menstrual cycle [73], with no PR-B detectable during the secretory and early proliferative phases. Similarly, Attia et al. [74] did not detect any PR-B protein in endometriotic tissue and they ascribe the clinically observed resistance of endometriosis to treatment with progestins to this absence of PR-B and to the presence of PR-A.

We next determined which steroid receptor(s) are involved in the response to MPA on the RANTES gene in the Ect1/E6E7 cell line, by using receptor-specific antagonists. Down-regulation of RANTES gene expression by MPA has previously been reported in endometrial stromal cells [33], and the PR was shown to mediate the effect. In addition, MPA has previously been shown to repress the proinflammatory chemokine IL-8 via the GR [31]. As we had shown that the Ect1/E6E7 cell line expresses AR, GR, PR and ER (Fig. 4B), we investigated the expression of the RANTES gene in the presence of OHF, the AR-specific antagonist, as well as RU486, the PR and GR antagonist. We did not investigate a role for the ER as evidence for activity of both MPA and NET-A via the ER is contradictory, with most of the literature indicating that these ligands do not bind to the ER [54,55,75]. The results show that in the Ect1/E6E7 cell line, RU486 behaves as an agonist, rather than as an antagonist (Fig. 5B). RU486 had previously been shown to act as both an agonist and as an antagonist, depending on receptor density [76]. Furthermore, it has been reported that RU486 is a partial agonist for both PR and GR when selective coregulators are recruited [77,78]. This approach of using RU486 as a receptor antagonist in this cell line is thus limited. However, when using the AR-specific antagonist OHF, we showed that the repression of MPA on the RANTES gene was lifted when the AR is antagonised, revealing a possible role for the AR (Fig. 5A). Since we showed that the endogenous AR is transcriptionally active in repression of a TNF-α-stimulated synthetic NFκB-reporter gene in the Ect1/E6E7 cell line (Fig. 7B), and since the RANTES promoter contains two NFκB binding sites which are strongly activated by TNF-α, [79], it seems likely that transrepression by MPA via the AR occurs via these NFκB sites. The fact that OHF could lift the repression of MPA on the RANTES gene to similar levels as the control (EtOH) suggests that the PR, despite being the most abundant receptor, as well as the GR, is not involved in the MPA-mediated down-regulation of the RANTES gene in the Ect1/E6E7 cell line. Consistent with this result, the androgenic properties of MPA have previously been proposed to be responsible for the anti-inflammatory effects of MPA on plasma inflammatory markers, such as C-reactive protein and serum amyloid A protein [80].

Considering that both MPA and NET-A have previously been reported to have androgenic properties [30,39,54–56,75,81–84], it was surprising that NET-A did not act via the AR in this cell line. However, as it is known that NET-A

can be metabolized [20], the possibility exists that NET-A is metabolized in this cell line and that the metabolite(s) are less efficacious AR agonists than NET-A itself. In addition, the repression of RANTES by MPA via the AR was not observed in the Vk2/E6E7 cell line, indicating that MPA acts in a cell-specific manner. As the levels of AR in the Ect1/E6E7 and Vk2/E6E7 cell lines are similar, it is unlikely that these differences in responses are due to different levels of steroid receptors. It may be due to other factors such as different extents of metabolism or even different levels of coregulators.

In summary, results from our study are the first to indicate not only differential regulation of the cytokine genes by Prog, MPA and NET-A, but also cell-specific effects. Moreover, we showed that the effect of MPA on the RANTES gene in the Ect1/E6E7 cell line is AR mediated. Further work is necessary to assess the impact of this novel finding. Moreover, the finding that MPA acts differently to NET-A, and does not mimic the effects of Prog, has important implications for women's health.

Finally, the immunoregulatory milieu of the genital mucosa is important in determining the response to antigenic challenge. Mucosal innate immune factors as well as pro-inflammatory cytokines are associated with elevated levels of HIV-1 shedding in the female genital tract [85]. Thus, a greater understanding of the immune responses at these different anatomical locations in the genital tract, and how MPA and NET used as contraceptives and in HRT regulate genes relevant to the cervicovaginal mucosal immune response, may have implications for women's risk of susceptibility to infections. Our results have important implications for choice of progestin [86] in contraception as well as the development of microbicides for the prevention of sexually transmitted diseases.

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