Antiretroviral drugs differentially modulate glucocorticoid activity via the glucocorticoid receptor *in vitro*

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Dissertation presented for the degree of

MASTER OF SCIENCE

In the Department of Molecular and Cell Biology

Faculty of Science

University of Cape Town

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June 2019

PLAGIARISM DECLARATION

I, Michael Kuipa declare the work presented herein is my original work, unless otherwise acknowledged. Neither this dissertation, nor part thereof has been, or will be submitted for another degree at any other university.

Signature: Date: 14 June 2019

Signed by candidate

ABSTRACT

Concurrent use of anti-retroviral drugs (ARVs) and progestin-based hormonal contraceptives is widespread. During times of stress and during glucocorticoid (GC) therapy, intracellular ARVs are in the presence of high concentrations of GCs, which regulate all aspects of immunity and inflammation via the glucocorticoid receptor (GR). However, the reciprocal modulation of ARV and steroid intracellular functions is relatively unexplored. In this study, the effects of the ARVs tenofovir disoproxil fumarate (TDF), dapivirine (DPV), and maraviroc (MVC) on activation of the GR and GR-regulated mRNA expression were investigated, in the absence and presence of select GR ligands. The effects of TDF and DPV on GR protein levels and phosphorylation were also determined. The inhibitory activity of these ARVs on HIV-1 infection in the presence of the progestins medroxyprogesterone acetate (MPA) and levonorgestrel (LNG), and a GR agonist, dexamethasone (DEX) was also assessed. This study shows that (0.01 nM–10 μM) TDF, DPV and MVC do not transactivate reporter gene expression via the unliganded GR exogenously expressed in the steroid receptor-deficient U2OS human osteosarcoma cell line, or alter the reporter gene transcriptional activity of (100 nM) MPA or LNG via the GR in these cells. However, (1 μM) TDF and DPV modulate the reporter gene transcriptional efficacy of (0.01 nM–10 μM) DEX via the GR. In the U2OS cell line model, (1 μM) TDF, but not DPV significantly decreased (1μM and 10μM) DEX-induced mRNA expression of the anti-inflammatory glucocorticoid-induced leucine zipper (GILZ) gene. TDF also appeared to decrease (1 μM) cortisol (CORT)- and MPA-induced GILZ mRNA expression. This may be mediated by the apparent increase in (100 nM and 1μM) DEX-induced phosphorylation at Serine 226 on the GR, observed in the presence of (1μM) TDF in this study. DPV and TDF (at 1μM) did not significantly alter GR protein levels, or cell-viability in the absence and presence of (100 nM) DEX, CORT or MPA in U2OS cells. However, (1 μM) DPV and TDF alone, significantly altered cell viability in peripheral blood mononuclear cells (PBMCs). In PBMCs, (1 μM) TDF, MVC and DPV alone altered basal GILZ mRNA expression and had variable, donor-specific effects on interleukin (IL)-6, IL-8, and interferon (IFN)-γ gene expression. In PBMCs from some of the nine donors tested, these ARVs had proinflammatory effects which may undermine their efficacy at preventing HIV-1 acquisition in pre-exposure prophylaxis products. Moreover, the ARVs proinflammatory effects may negatively impact HIV-1 disease progression and increase the risk of non-AIDS mortality in individuals using the ARVs therapeutically. Neither (1 μM) DPV, TDF nor MVC significantly

altered the effects of (100 nM) DEX on the immunomodulatory genes assessed in PBMCs. DEX, MPA and LNG (at 100 nM) did not affect the anti-HIV-1 activity of the ARVs (at 1 μM) in PBMCs from the majority of the three donors tested in this study. Taken together, the results show that ARVs can modulate GR activity in an ARV-, steroid-, gene- and cell-specific manner, while the steroids investigated did not modulate ARV anti-HIV-1 activity.

LIST OF ABBREVIATIONS

A	amps
AIDS	Acquired Immune Deficiency Syndrome
AKT	Ak strain transforming
AMP	adenosine monophosphate
AMPK	adenosine 5' monophosphate-activated protein kinase
ANOVA	analysis of variance
AP-1	activator protein 1
AR	androgen receptor
ARV	anti-retroviral **drug**
ASPIRE	A Study to Prevent Infection with a Ring for Extended Use
ATCC	American type culture collection
ATP	adenosine triphosphate
AVERT	AIDS Virus Education Research Trust
BMD	bone mineral density
bp	base pair
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CBP	CREB binding protein
CCL	chemokine (C-C motif) ligand
CCR5	chemokine receptor type 5
CD	cluster of differentiation
CDK	cyclin dependent kinase
cDNA	complementary DNA
CREB	cyclic AMP response element-binding protein
cs	charcoal stripped
CTL	cytotoxic lymphocyte
COC	combined oral contraceptive
CXCR4	C-X-C chemokine receptor type 4
DBD	DNA binding domain
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DEPC	diethylpyrocarbonate

DEX	dexamethasone
DHT	dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DMPA	depot-medroxyprogesterone acetate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DPV	dapivirine
DREAM	Dapivirine Ring Access and Monitoring
E_2	estradiol/estrogen
EC	epithelial cell
ECL	enhanced chemiluminescence
EC50	effective concentration required for 50% of maximal response
EDTA	ethylenediaminetetra-acetic acid
EFV	efavirenz
ELISA	enzyme-linked immunosorbent assay
Env	HIV envelope protein
ER	estrogen receptor
ETG	etonogestrel
EtOH	ethanol
FACTS	Follow-on African Consortium for Tenofovir Studies
FBS	fetal bovine serum
FDA	U.S. Food and Drug Administration
FGT	female genital tract
FI	fusion inhibitor
FSH	follicle stimulating hormone
FTC	emtricitabine
g	earth's gravitational force
GAPDH	glyceraldehyde phosphate dehydrogenase
GILZ	glucocorticoid induced leucine zipper
GMP	guanosine monophosphate
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GSK	glycogen synthase kinase

HAART	highly active antiretroviral therapy
HBD	human beta defensin
HC	hormonal contraception
HESN	highly HIV-1 exposed seronegative
HIV-1	human immunodeficiency virus subtype 1
HLA	human leukocyte antigen
HOPE	HIV Open Label Prevention Extension
HPV	human papilloma virus
HRT	hormone replacement therapy
Hsp	heat shock protein
HSV	herpes simplex virus
IκB-α	inhibitor kappa B alpha
IC50	inhibitory concentration that reduces the maximal response by 50%
IHC	injectable hormonal contraceptives
IL	interleukin
INI	integrase inhibitor
IP-10	interferon-gamma-inducible protein 10
IPM	International Partnership for Microbicides
IU	infectious units
IUD	intrauterine device
IVR	intravaginal ring
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LABA	long-acting β(2)-adrenoreceptor agonist
LB	luria broth
LBD	ligand binding domain
LH	luteinising hormone
LNG	levonorgestrel
LPS	lipopolysaccharide
LTR	long terminal repeat
M	Molar
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein 1
min	minutes

MIP	macrophage inflammatory protein
MKP-1	MAPK phosphatase-1
MOPS	4-morpholine-propanesulfonic acid
MPA	medroxyprogesterone acetate
MPT	multipurpose prevention technology
mRNA	messenger RNA
MR	mineralocorticoid receptor
MTN	Microbicide Trials Network
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MVC	maraviroc
NET	norethisterone
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
NK	natural killer cell
NNRTI	non-nucleotide reverse transcriptase
NRTI	nucleotide reverse transcriptase
NTD	amino-terminal domain
OAS	oligoadenylate synthetase
P_4	progesterone
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI	protease inhibitor
PR	progesterone receptor
PrEP	pre-exposure prophylaxis
PRR	pattern recognition receptor
RT-qPCR	reverse transcription quantitative PCR
RANK	receptor activator of NF-κB
RANKL	receptor activator of NF-κB ligand
RANTES	regulated upon activation, normal T cell expressed and secreted
RBA	relative binding affinity
RLU	relative light units
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute

RU486	mifepristone
SANAC	South African National AIDS Council
SDS	sodium dodecyl sulphate
Ser	serine
SGK-1	serum/glucocorticoid regulated kinase 1
SIV	simian immunodeficiency virus
SLIPI	secretory leukocyte protease inhibitor
SR	steroid receptor
SRC	steroid receptor co-activator
SRE	steroid response element
STI	sexually transmitted infection
TAF	tenofovir alafenamide
TBS	TRIS-buffered saline
TBST	TRIS-buffered saline-tween
TDF	tenofovir disoproxil fumarate
TFV	tenofovir
TFV-DP	tenofovir diphosphate
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumour necrosis factor
Treg	regulatory T cell
U	international units
UN	United Nations
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organisation
Wnt	wingless/integrated
w/v	weight per unit volume
v/v	volume per unit volume
V	Volts
VOICE	Vaginal and Oral Interventions to Control the Epidemic

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CHAPTER 1

LITERATURE REVIEW

1.1 *HIV-1 prevalence, and incidence of unintended pregnancy in Sub-Saharan Africa*

In 2017, approximately 36.9 million people globally were living with HIV/AIDS (Joint United Nations Programme on HIV/AIDS (UNAIDS), 2018). Although only 6% of the global population inhabits East and Southern Africa, over half of those infected (19.4 million people in 2017) reside in this region (UNAIDS, 2018). South Africa has the highest HIV-1 prevalence globally; with approximately 7.2 million people infected, it accounts for a third of all new infections in southern Africa (UNAIDS, 2018).

Women and adolescent girls are disproportionately affected by the epidemic (**Fig. 1.1**). In Sub-Saharan Africa, 75% of new infections among adolescents aged 15–19 years are in girls, and young women aged 15–24 years are twice as likely to be living with HIV than men (UNAIDS, 2018). In South Africa, women aged 15–24 years accounted for 37% of new infections in 2016 (South African National AIDS Council (SANAC), 2017). Socio-economic factors which disadvantage women, and physiological differences which render women more susceptible to HIV-1 acquisition than men are thought to be responsible (Greig *et al.*, 2008). Innate and adaptive immune responses in men and women differ, due to the actions of the sex steroid hormones progesterone (P_4) and estrogen (E_2) in women, and testosterone in men. Sex chromosome genes also contribute to sex-based immunological differences (reviewed by Klein and Flanagan, 2016). A further burden on women are unintended pregnancies, which too occur with the highest incidence in Sub-Saharan Africa (Sedgh *et al.* 2014). The consequences are often infant and maternal morbidity or mortality, unsafe abortions and, in HIV-1 positive women, mother-to-child transmission of the virus (Sedgh *et al.* 2014). As such, the use of hormonal contraceptives (HCs), for effective timing of pregnancy and family planning is widespread in the region (UN, 2015).

As evidenced by the data presented here, there is a need for HIV-1 preventative strategies, targeted at women. Concurrently, there is a need for interventions which reduce the burden of unintended pregnancy, and to offer women control over their reproductive health. There are efforts underway to develop effective HIV-1 preventative interventions, targeted at women. Microbicides are topical products, applied intravaginally, to prevent the early events of heterosexual HIV-1 transmission

(Kelly and Shattock, 2011). The most clinically advanced microbicides are based on antiretroviral drugs (ARVs) (Verma *et al.*, 2011). Multipurpose prevention technologies (MPTs) are ARV and HC combination products for the simultaneous prevention of unintended pregnancy and HIV-1 acquisition in women (reviewed by Friend *et al.*, 2013; Jean-Pierre *et al.*, 2016).

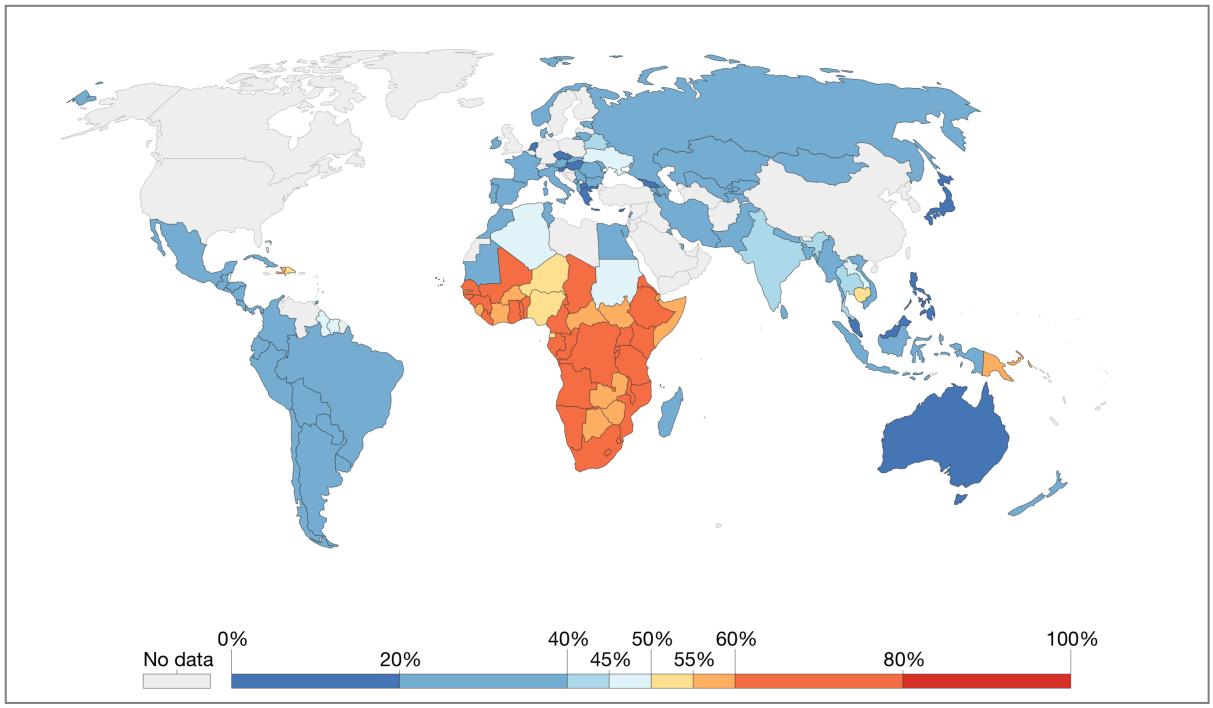

Figure 1.1: HIV-1 prevalence is higher in women in Sub-Saharan Africa . Female rate is shown as a percentage of the population infected with HIV-1 (aged 15 or older) in 2017. Data was published by World Bank at http://data.worldbank.org/data-catalog/world-development-indicators, and is based on UNAIDS estimates (UNAIDS, 2018). Figure is adapted from https://ourworldindata.org/hiv-aids#hiv-aids-and-gender.

1.2 *Progestins in common hormonal contraceptives*

HCs differ in their mode of delivery (oral, intramuscular or subcutaneous injection, transdermal patch or intravaginal implant), the frequency with which they are administered, as well as the type and dose of progestogen they contain (reviewed by Stanczyk *et al.*, 2013; Hapgood *et al.*, 2018). Progestogens are defined as compounds which alter the state of the endometrium of the uterus, from secretory to proliferative, thereby preventing ovulation and pregnancy (Stanczyk and Henzl, 2001). Ovulation in women is inhibited by high levels of P_4, the endogenous progestogen (reviewed by Stanczyk and Henzl, 2001). Progestins are synthetic progestogens which, like P_4, exert their progestogenic effects via the progesterone receptors (PR) (reviewed by Stanczyk *et al.*, 2013; Hapgood *et al.*, 2014a). Depending on their chemical structure, they may bind to other

members of the nuclear receptor family, acting as agonists, partial agonists or antagonists (Stanczyk *et al.*, 2013; Hapgood *et al.*, 2014a).

Long-acting injectable hormonal contraceptives (IHCs) are the most widely used form of contraception in Sub-Saharan Africa, with approximately 16.5 million women using IHCs in 2015 (UN, 2015). Although protective against unintended pregnancy, they are not protective against sexually transmitted diseases (unlike the condom) (Baeten *et al.*, 2001). IHCs are favoured in developing countries because of the relatively long interval between injections and because they allow for discreet usage, without the need for partner co-operation (Affandi, 2002).

1.2.1 *Medroxyprogesterone acetate*

The most common IHC used in southern Africa is 150 mg medroxyprogesterone acetate (MPA), administered as an intramuscular injection at three monthly intervals as Depo-Provera or Depot-MPA (DMPA) (Affandi, 2002). MPA is structurally related to P_4 (Stanczyk, 2003), and has been used for more than 30 years, both in HCs and for postmenopausal hormone replacement therapy (HRT) (Stanczyk *et al.*, 2013). Reported peak serum concentrations for DMPA are relatively high compared to progestins used in many other forms of HC, and vary between individuals and between studies (Hapgood *et al.*, 2018). Inconsistency in time post-injection at which serum concentrations are determined, number of injections and experimental methodologies between studies are likely to account for many of the discrepancies between studies (Hapgood *et al.,* 2018). Peak serum concentrations are typically reported in the range of 10–40 nM following an intramuscular injection (see **Table 1.1**) (Kirton and Cornette, 1974; Virutamasen *et al.*, 1996; Fang *et al.*, 2004), and concentrations as high as 100 nM have been reported in some users (Koetsawang, 1977). MPA serum concentrations typically plateau to approximately 2.6 nM (Mishell, 1996).

MPA use is associated with several adverse effects, including a loss in bone mineral density (BMD) and increased risk of breast cancer (Hapgood *et al.*, 2018). Moreover, meta-analysis of higher quality observational and clinical studies suggests MPA, unlike other HCs, significantly increases the risk of HIV-1 acquisition by 1.4-fold (Polis *et al.*, 2016). It is important to note that the interpretation of the results of observational studies is confounded by factors like condom usage and frequency of sexual acts, which, in these studies, are difficult to control for (Hapgood *et al.*, 2018). A growing body of clinical, animal and *ex vivo* studies, recently reviewed comprehensively by Hapgood *et al.*, support biological mechanisms consistent with an increased risk of HIV-1

infection in MPA users (Hapgood *et al.*, 2018). These mechanisms include increasing the risk of acquiring other sexually transmitted infections (including chlamydia and herpes simplex virus type 2 (HSV-2) (Deese *et al.*, 2018), enhanced activation and recruitment of HIV-1 target cells to the FGT through modulation of cytokine and chemokine expression and enhanced HIV-1 entry and replication within these cells (Hapgood *et al.* 2018). Additional mechanisms include increased genital injury signatures, alterations to the vaginal microbiota and impaired integrity of the cervicovaginal mucosa (Hapgood *et al.* 2018).

Like other progestins, MPA has a high affinity for the PR. As steroid receptors have structurally related ligand binding domains (LBDs), dependent on their structure, progestins are able to bind to other members of the steroid receptor family (Stanczyk *et al.*, 2013; Hapgood *et al.*, 2014a). Whereas binding to the ER has not been reported, progestins have varying affinities for the androgen receptor (AR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Stanczyk *et al.*, 2013; Hapgood *et al.*, 2014a). Unlike other progestins, MPA has a relatively high affinity for the GR, binding with a higher affinity than both P_4 and cortisol (CORT), the endogenous ligand for the GR. Like many other progestins, MPA exhibits binding to the AR (Stanczyk *et al.*, 2013; Hapgood *et al.*, 2014a). However, binding affinity does not equal biological activity, which is determined by the conformation the ligand induces in the receptor LBD and other receptor domains, which influence transcriptional co-regulator recruitment and transcriptional activity (Ronacher *et al.*, 2009). Nevertheless, MPA has been shown to be a relatively potent partial agonist for upregulation of several GR-regulated genes, and a relatively potent full agonist for the downregulation of GR-regulated genes, including proinflammatory cytokines and chemokines (Koubovec *et al.*, 2004, 2005; Govender *et al.*, 2014; Hapgood *et al.*, 2014b).

1.2.2 *Levonorgestrel*

Levonorgestrel (LNG) is a progestogen structurally related to testosterone, and is the biologically active form of norgestrel (Stanczyk, 2003). LNG is delivered in intrauterine devices (IUDs), trans-epidermal implants and combined oral contraceptive (COC) pills (Stanczyk, 2003). LNG peak serum concentrations are typically in the range of 0.3–28 nM, and are dependent on the mode of administration (see **Table 1.1**) (Sivin *et al.*, 1997; Licea-Perez *et al.*, 2007; Hidalgo *et al.*, 2009). It should be noted that serum levels required for effective contraception have not been established for most progestins, including MPA and LNG (Hapgood *et al.* 2018). Unlike MPA, LNG exhibits

no binding affinity for the GR, or transcriptional activity via the GR, but does bind to the AR (Stanczyk *et al.*, 2013; Hapgood *et al.*, 2014a). MPA and LNG have been reported to increase genital permeability in mice, which was associated with a decrease in cell-adhesion molecules, as well as an influx of inflammatory cells into the genital tract (Quispe Calla *et al.*, 2016). Quispe Calla *et al.* also reported that LNG and MPA increased the risk of vaginal HSV-2 transmission in these mice (Quispe Calla *et al.*, 2016). Additional basic and clinical studies are required to determine the effects of MPA and LNG on genital tract barrier function in humans.

Table 1.1: **MPA and LNG mode of administration, peak serum concentrations and relative binding affinities to steroid receptors.**

Progestin	Mode of administration	Peak serum concentration (nM)	Steroid receptor relative binding affinities (% relative binding affinity)[•10]
MPA	3 monthly injection:		**AR**: 151 **ER**: – **GR**: 74 **PR**: 65–98 **MR**: 0.13
	Intramuscular (DMPA-IM)	10–40[1–3]	
	Subcutaneous (DMPA-SQ)	2.5–4[4–6]	
LNG	1 year IUD 3 or 5 year implant Daily COC pills Emergency contraceptive pills	0.3–28[7–9]	**AR**: 58 **ER**: – **GR**: 1–7.5 **PR**: 23–96 **MR**: –

References: [1] Kirton and Cornette, 1974; [2] Virutamasen *et al.*, 1996; [3] Fang *et al.*, 2004; [4] Jain *et al.*, 2004; [5] Toh *et al.*, 2004; [6] Halpern *et al.*, 2014; [7] Sivin et al., 1997; [8] Licea-Perez *et al.*, 2007; [9] Hidalgo *et al.*, 2009; [10] Hapgood *et al.*, 2014a. Symbols; [•] Relative binding affinities are relative to 100% for reference steroids for each steroid receptor, which were as follows: PR, P_4; AR, dihydrotestosterone; GR, dexamethasone; MR, aldosterone and ER, E_2.

1.3 *The female genital mucosa and HIV-1 infection*

HIV-1 transmission in women typically occurs at the mucosa of the FGT (reviewed by Hladik and McElrath, 2008; Roan and Jakobsen, 2016). Here, several mechanisms are protective against HIV-1 infection (reviewed by Wira and Fahey, 2004; Wira *et al.*, 2005; Reis Machado *et al.*, 2014), as evidenced by the relatively low transmission rate per vaginal sex act (reported to be as low as 0.07–0.08%) (Boily *et al.*, 2009). To establish successful infection, HIV-1, which is present in male ejaculate, must withstand several innate and adaptive immune mechanisms in the FGT, traverse the genital epithelium, and establish infection in the underlying lamina propria in cluster of differentiation (CD)4+ target cells (Hladik and McElrath, 2008).

1.3.1 *Mechanisms of innate immune protection in the female genital tract*

Continuously sloughed stratified squamous epithelium provides an effective barrier against HIV-1 invasion in the lower reproductive tract (vagina and ectocervix) (Wira and Fahey, 2004). In contrast, tight junctions between single-layered columnar epithelial cells (ECs) in the upper reproductive tract (endocervix, endometrium and fallopian tubes) are a less robust means of mechanical protection (Wira and Fahey, 2004). Innate immune cells; ECs, macrophages, dendritic cells (DCs) and natural killer (NK) cells, recognise foreign pathogens through pattern recognition receptors (PRRs)–germ-line encoded sensors which recognize conserved pathogen-associated molecular patterns expressed by microorganisms (Akira *et al.*, 2006). PRRs can be divided broadly into two groups; toll-like receptors (TLRs), which are expressed on cell surfaces, or found in endosomes (TLRs have recently been reviewed extensively by Vidya *et al.*, 2018), and intracellular nucleic acid sensors found in the cytoplasm (reviewed by Sparrer and Gack, 2015). HIV-1 components recognised by PRRs include its single stranded (ss)RNA genome and the reverse transcribed DNA intermediates produced early during infection (reviewed by Jakobsen *et al.*, 2015). Secondary structured HIV-derived RNAs are recognised by retinoic acid-inducible gene I (Solis *et al.*, 2011), and HIV-1 DNA is recognised by cyclic guanosine monophosphate-adenosine monophosphate synthase (Gao *et al.*, 2013).

Innate immune cells secrete microbial compounds which contribute to preventing pathogenic infection. These include lysozyme, cytokines and chemokines (including interleukin (IL)-6, IL-8 and macrophage inflammatory protein (MIP)-1β), as well as defensins (reviewed by Wira *et al.,* 2005). Defensins are antimicrobial peptides with broad anti-HIV-1 activity, achieved through blocking viral entry into cells, interfering with viral replication, direct viral inactivation and increasing the production of other antiviral factors (the role of defensins in HIV-1 pathogenesis has been reviewed by Pace *et al.*, 2017). Pro-inflammatory cytokines and chemokines are chemo-attractants for neutrophils, monocytes and T cells, which enhance inflammation and destroy invading pathogens through cytotoxic killing or phagocytosis (Wira *et al.,* 2005). HIV-1 infection is profoundly influenced by the effects of cytokines and chemokines, which may inhibit HIV-1 infection (as do interferon (IFN)-α (Shirazi and Pitha, 1992), IFN-β (Gessani *et al.*, 1994), macrophage inflammatory protein (MIP)-1α and MIP-1β (Cocchi *et al.*, 1995)), stimulate HIV-1 infection (for example, macrophage colony-stimulating factor (Kalter *et al.*, 1991) and IL-1 (Osborn *et al.*, 1989)) or have both inhibitory and stimulatory effects, which are dependent on their concentration, the presence of other cytokines and the point during infection at which they

are present (examples include IL-4 (Kazazi *et al.*, 1992; Wang *et al.*, 1998), IL-6 (Kazazi *et al.*, 1992; Wang *et al.*, 1998), IL-8 (Nagira *et al.*, 1999; Lane *et al.*, 2001), tumour necrosis factor (TNF)-α (Osborn *et al.*, 1989; Lane *et al.*, 1999) and IFN-γ (Kornbluth *et al.*, 1990; Roff *et al.* 2013)). Innate and adaptive immune responses in the FGT are under the control of P_4 and E_2, the production of which varies cyclically over the course of the menstrual cycle (reviewed by Wira *et al.*, 2005). Protection therefore varies depending on the menstrual cycle; during the secretory phase of the cycle (when P_4 levels are relatively high and E_2 levels are relatively low), immune responses are dampened to support pregnancy and fertilization, increasing susceptibility to STIs, including HIV-1 (reviewed by Wira *et al.*, 2014).

The main HIV-1 target cells are DCs, macrophages and T cells (Hladik and McElrath, 2008). HIV-1 infection requires interaction with host CD4 receptors, which are expressed on the surface of the aforementioned cells (Wyatt and Sodroski, 1998). DCs are a primary HIV-1 target and contribute greatly to dissemination of the virus to submucosal cells and subsequently, associated lymph nodes (reviewed by Wu and KewalRamani, 2006). Based on experiments in cervical explants, DCs have been predicted to account for up to 90% of HIV-1 dissemination (Hu *et al.*, 2004). DCs are antigen-presenting cells, which constantly take up extracellular fluid, antigens and pathogens by micropinocytosis, endocytosis and phagocytosis respectively (the immunology of DCs is reviewed by Banchereau *et al.*, 2000). C-type lectins, expressed on the surface of DCs, attach to HIV-1 envelope glycoprotein, and are the principle means of HIV-1 capture (Banchereau *et al.*, 2000; Wu and KewalRamani, 2006). Infection of CD4+ T cells, which are the primary targets of HIV-1 infection, and account for up to 60% of immune cells in the FGT (Givan *et al.* 1997), occurs through an infectious synapse (Garcia *et al.*, 2005), trans-infection through DC-derived exosomes (Wiley and Gummuluru, 2006) or by *cis*-infection with HIV-1 virions released following replication in DCs (Kawamura *et al.*, 2003). Macrophages, like CD4+ T cells, are productively infected by HIV-1, and, due to their longer lifespan following infection, act as long-term reservoirs of HIV-1 infection (Sharova *et al.*, 2005; Waki and Freed, 2010). Like DCs, macrophages disseminate HIV-1 to CD4+ T cells by cell-cell transmission through infectious synapses or by *cis*-infection (Waki and Freed, 2010).

1.3.2 *HIV-1 replication in target host cells*

An intricate, multistep process involving interaction of HIV envelope gp160 glycoproteins with host receptors facilitates the entry of HIV into its target cells (Wilen *et al.*, 2010; Wyatt and

Sodroski, 1998). HIV gp160– initially synthesized as a polyprotein and subsequently proteolytically cleaved–is composed of gp120; an exterior envelope glycoprotein non-covalently bound to gp41, which spans the viral membrane (Wilen *et al.*, 2010; Wyatt and Sodroski, 1998). HIV gp120 binds to the surface of cells via the CD4 receptor, whereas gp41 facilitates the fusion of viral and cellular membranes (Wilen *et al.*, 2010; Wyatt and Sodroski, 1998). The binding of gp120 to the CD4 receptor induces a conformational change which exposes the gp120 coreceptor binding site (Wilen *et al.*, 2010; Wyatt and Sodroski, 1998). Subsequently, gp120 binds to its coreceptor; either the chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) (Wu et al., 1996; Berger et al., 1999). Virus which preferentially utilizes CCR5 as its coreceptor is termed (CC)R5 tropic whereas virus which utilizes CXCR4 as its coreceptor is termed X4 tropic (or CXCR4 tropic) (Berger *et al.*, 1998). The greater proportion (more than 95%) of transmitted HIV strains are R5 tropic (Margolis and Shattock, 2006). Binding of gp120 to its coreceptor triggers a conformational change in gp41, which exposes a hydrophobic fusion protein that facilitates fusion of viral and host membranes (Doms and Trono, 2000). HIV-1 gp120 and gp41 are recognised by the PRRs TLR10 (Henrick *et al.*, 2019) and TLR2, respectively (Henrick *et al.*, 2015).

Subsequently, the viral core, containing the ssRNA genome is released into the host cytoplasm (Wilen *et al.*, 2010). HIV-1 reverse transcriptase in translated (by free cytoplasmic ribosomes) as part of the Gag-Pol polyprotein, which consists of *gag* (structural capsid, nucleocapsid and matrix proteins) and *pol* (reverse transcriptase, integrase and protease) (Li *et al.*, 1992). The ssRNA genome is reverse transcribed into dsDNA, translocated into the nucleus and integrated into the host chromosome in a process mediated by viral integrase (Craigie and Bushman, 2012). The integrated genome is transcribed into viral RNA, which is exported to the cytoplasm and translated (Sundquist and Kräusslich, 2012). The resulting products are assembled into virions at the plasma membrane (Sundquist and Kräusslich, 2012). Virions bud off from the cell surface, obtaining their lipid envelope, after which HIV-1 protease mediates maturation of the virions (Sundquist and Kräusslich, 2012). Matured virions are able to infect new host target cells (Sundquist and Kräusslich, 2012).

1.3.3 *HIV-1 disease progression*

Immediately following HIV-1 transmission, during the eclipse period of infection, HIV-1 replicates in the mucosa, submucosa and associated lymph tissues close to the site of transmission, and is undetectable in the plasma (Cohen *et al.*, 2011). Acute HIV-1 infection is the earliest

detectable phase of infection (Haase, 1999; Cohen *et al.*, 2011). It is characterized by a surge in plasma viremia, as HIV-1 rapidly disseminates into, and replicates in gut-associated lymphoid tissue (Brenchley *et al.*, 2004; Brenchley and Douek, 2008). The gut contains approximately 60% of the body's T cells (Guy-Grand and Vassalli, 1993), and undergoes the greatest depletion of these cells through all stages of HIV-1 infection (Brenchley *et al.*, 2004). Due to constant exposure to foreign antigens and proinflammatory cytokines, a large proportion of CD4+ T cells in the gut are activated, and express CCR5 (Unutmaz *et al.*, 1994; Brenchley *et al.*, 2004; Brenchley and Douek, 2008). Importantly, upon activation, T cells are generally destined to undergo activation-induced cell death (reviewed by Green *et al.*, 2003). Both uninfected and infected T cells die during HIV-1 infection; the latter undergo a programmed cell death associated with intense inflammation and cytoplasmic expulsion termed pyroptosis (Doitsh *et al.*, 2014). Alternatively, activation of DNA-dependent protein kinase in response to viral integration (Cooper *et al.*, 2013), or the expression of cytotoxic HIV-1 protease (Ventoso *et al.*, 2005) induce death in infected T cells. Uninfected T cells, which account for the most T cell death (Finkel *et al.*, 1995), die either as a result of activation-induced cell death, the cytopathic effects of HIV-1 proteins or the over expression of death-ligands (reviewed by Cummins and Badley, 2014). Immune responses mounted in the lamina propria of the gut in response to HIV-1 infection result in villous atrophy and enterocyte apoptosis (Batman *et al.*, 1989), which, along with HIV-induced CD4+ T cell depletion and immunodeficiency, results in a loss of the structural and immunological integrity of the gut (Brenchley *et al.*, 2004; Brenchley and Douek, 2008). With a loss in integrity, microbial translocation occurs; a process in which gut microbial products are exposed systemically, activate immune cells, and stimulate proinflammatory cytokine production (Brenchley *et al.*, 2006).

Acute HIV-1 infection is also characterized by a cytokine storm–a surge in cytokine and chemokine production by innate immune cells–which also promotes systemic immune activation and inflammation (Stacey *et al.*, 2009). In an effort to restore the body's CD4+ T cell balance, the immune system triggers the activation and proliferation of remaining CD4+ T cell pools, which, rather than replenishing CD4+ T cells, drives HIV-1 disease progression by increasing the number of viral targets (Douek *et al.*, 2003). In addition to activation by HIV-1 antigens, HIV-1 proteins have also been shown to induce T cell activation (Abbas and Herbein, 2013). During the acute phase of infection a reservoir of latently infected CD4+ T cells is established (Chun *et al.*, 1998), and an HIV-1 specific cellular immune response develops (Koup *et al.*, 1994). Long-lived central memory CD4+ T cells are thought to be the most important reservoir for latent HIV-1 (Brenchley *et al.*, 2004). Other cell types including ECs, fibrocytes and astrocytes have been suggested to

contribute to the latent reservoir, but it is uncertain whether this is the case *in vivo* (reviewed by Kandathil *et al.*, 2016).

Following the acute phase of infection, HIV-1 infection enters into an asymptomatic, chronic phase, which may last several years (reviewed by Ford *et al.*, 2009). It is characterized by a progressive decline in the remaining CD4+ T cells, and chronic immune activation (Ford *et al.*, 2009). Due in part to HIV-1 specific immune responses, and the massive attrition of CD4+ T cells during the acute phase of infection, viral loads during this period may be up to a 1000-fold lower than during the acute phase of infection (reviewed by Haase, 1999; Douek *et al.*, 2003; Boasso and Shearer, 2008). During this period, HIV-1 viral replication persists, albeit in a small number of activated CD4+ T cells and both CD4+ and CD8+ T cells are chronically activated (Haase, 1999; Brenchley *et al.*, 2004). HIV-associated chronic immune activation is associated with transforming growth factor (TGF)-β production in secondary lymphoid tissues, which induces collagen deposition and fibrosis, eventually destroying these sites of T cell production (Schacker *et al.*, 2002). Moreover, chronic immune activation directly inhibits the function of B cells NK cells, DCs and monocytes, impairing their ability to control viral replication, which compounds immune activation (reviewed by Muller-Trutwin and Hosmalin, 2005; Moir and Fauci, 2009). Over the course of HIV-1 infection, in approximately 50% of individuals, an HIV-1 phenotypic switch from R5 virus to X4 virus occurs (Berger *et al.*, 1999; Regoes and Bonhoeffer, 2005; Mosier, 2009). The reasons behind this are unclear, but it has been proposed that R5 viruses have higher fitness for transmission, or that conditions in the host, for example the proportion of T cells expressing either coreceptor, change to favor X4 virus over the course of disease progression (Regoes and Bonhoeffer, 2005; Kamp, 2009; Mosier, 2009). Immunodeficiency, established during the acute phase of infection, supports the reactivation of latent infections, like the highly prevalent cytomegalovirus (CMV) or influenza, which further induce immune activation and inflammation (Doisne *et al.*, 2004). Evidently, chronic immune activation and inflammation are central to the pathogenesis of HIV-1 infection, and drive a vicious cycle in which HIV-1 infection induces immune activation, inflammation and immune dysfunction, which in turn advance infection (Brenchley *et al.*, 2004, Brenchley *et al.*, 2006; Brenchley and Douek, 2008; Ford *et al.*, 2009).

Besides progressive immunodeficiency, HIV-1-induced chronic immune activation and inflammation are associated with other health complications (reviewed by Deeks, 2011). Notably, numerous studies have shown HIV-1 infection is associated with an increased risk of cardiovascular and hepatic disease (Currier *et al.*, 2008; Smith *et al.*, 2014). On reaching the liver,

microbial products released following the destruction of the gut may induce a cascade of immune cell activation, as well as the release of coagulation factors which increase the risk of blood clotting, and contribute to liver dysfunction, reduced protein synthesis and reduced microbial clearance (Deeks, 2011). In this way, microbial translocation increases the risk of liver fibrosis and cardiovascular disease (Deeks, 2011). HIV-1 infection is also associated with increased inflammation in arteries, concurrent with increased markers of macrophage activation (Burdo *et al.*, 2011). Some inflammatory cytokines have been implicated in the development of coronary heart disease (Yudkin *et al.*, 2000; Crowe *et al.*, 2010). For example IL-6, which is elevated in individuals infected with HIV-1 (Funderburg *et al.*, 2013), and is associated with atherosclerosis and coronary disease, reduces the expression of lipases which metabolize circulating triglycerides and increases the uptake of lipids by macrophages, (Yudkin *et al.*, 2000). Moreover, systemic inflammation induces the secretion of chemoattractant cytokines in endothelial cells on the interior surface of blood vessels, which enhances migration of macrophages into blood vessels, and inhibits their emigration out of them (Westhorpe *et al.*, 2009). Inflammation also promotes the expression of adhesion factors, in the endothelium and on macrophages and monocytes (Murphy *et al.*, 2008). Atherosclerosis, which may lead to coronary disease, develops when activated macrophages and monocytes infiltrate the endothelium of blood vessel walls, where they adhere, phagocytize lipids and other toxins, and form foam cells, leading to the formation of atherosclerotic plaques (Crowe *et al.*, 2010). When plaques are dislodged or rupture, the coagulation process is activated, and blood vessels are blocked, resulting in tissue damage (reviewed by Bentzon *et al.*, 2014).

1.3.4 *Genital inflammation is a risk factor for HIV-1 acquisition*

Genital inflammation is associated with an increased risk of sexual HIV-1 acquisition (Passmore *et al.*, 2016). Major causes of genital inflammation include changes to the vaginal microbiome and pre-existing STIs (which may be asymptomatic) (reviewed by Passmore *et al.*, 2016). In the Centre for the AIDS Programme of Research in South Africa (CAPRISA) 004 trial, which assessed the effectiveness of a 1% tenofovir (TFV) gel at preventing HIV-1 acquisition, the presence of genital inflammatory cytokines, including MIP-1α, MIP-1β, and IL-8, in cervicovaginal lavages from women who participated in the trial was shown to be associated with HIV-1 seroconversion (Masson *et al.,* 2015). Masson *et al.* also showed that the presence of genital inflammation in HIV-1 seroconvertors was associated with viruses of lower replicative fitness establishing productive HIV-1 infection (Masson *et al.* 2015). Subsequent post-hoc analysis of the levels of

proinflammatory cytokines in cervicolavages from women involved in the CAPRISA 004 trial found, in women without genital inflammation, TFV was 57% protective against HIV-1, but was only 3% protective in women with significantly elevated levels of genital proinflammatory cytokines (Mckinnon *et al.*, 2018). When systemic cytokine and immune mediators in women in the CAPRISA 004 trail were assessed, it was concluded that innate immune activation, through activation of cytokine and NK cell immune mediators, individually or in combination, was associated with HIV-1 acquisition (Naranbhai *et al.*, 2012). Naranbhai *et al.* suggested addition of a suppressor of innate immunity to the TFV gel as a potential means of enhancing its effectiveness (Naranbhai *et al.*, 2012). Early failed microbicide candidates cellulose sulfate and nonoxynol-9 enhanced IL-1α and IL-8 expression and activated the NF-κB pathway, which was proposed to have caused inflammation in the FGT, as well as the interruption of tight junctions. All these mechanisms have been suggested to be involved in the enhancement of HIV-1 acquisition (Fichorova, 2004a, 2004b; Mesquita *et al.*, 2009).

1.3.5 *Immune quiescence may be protective against HIV-I infection*

A phenotype of low immune activation systemically, and in the FGT, has been shown to be protective against HIV-1 infection (reviewed by Card *et al.*, 2013). While HIV-1 can infect non-activated T cells, viral replication is inefficient in these cells as a large number of host factors essential for HIV-I replication are expressed preferentially in activated T cells (Vatakis *et al.*, 2010). Moreover, some T cell subsets are more prone to infection with HIV-1 than others–in the human FGT, Th17 cells have been reported to be most susceptible to HIV-1 infection (Rodriguez-Garcia *et al.*, 2014). Similar findings have been reported in a macaque model of SIV infection (Stieh *et al.*, 2016). It may be that the proportion of Th17 cells at mucosal sites varies between individuals and predicts infectability, but this remains to be determined. Immune quiescence is implicated in protection in several highly HIV-1 exposed seronegative (HESN) cohorts, who demonstrate natural immunity to HIV-1 (Card *et al.*, 2013). Microarray analysis of blood from HESN female sex workers in Nairobi found NK cell cytotoxicity and T cell receptor signalling pathways were more downregulated compared to HIV-1 negative controls, suggesting immune quiescence was a mechanism of protection in these individuals (Songok *et al.*, 2012). Furthermore, in another cohort, T cells from HESN individuals were determined to express lower levels of immune activation markers, compared to another group of individuals who underwent seroconversion over the course of the study (Koning *et al.*, 2005). Moreover, HESN individuals have higher proportions of regulatory T cells (Tregs) (Fowke *et al.*, 2012), which are an immunosuppressive subpopulation

of T cells that suppress the proliferation and activation of effector T cells, as well as cytokine production (the immunobiology of Tregs has been reviewed by Kondelkova *et al.*, 2010).

1.4 *Antiretroviral therapy*

Although a functional cure for HIV/AIDS is not available, the use of combination highly active antiretroviral therapy (HAART) has significantly extended the life expectancy of HIV-1 positive individuals by decades, when correctly adhered to (reviewed by Arts and Hazuda, 2012). HAART involves the use of three or more antiretroviral drugs (ARVs), which target two or more stages in the HIV-1 replication cycle, thus forestalling drug resistance (Arts and Hazuda, 2012). In Southern Africa, the preferred first-line ARV treatment regimens are tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC), in combination with either efavirenz (EFV), dolutegravir or ripivirine (Meintjes *et al.*, 2017). In South Africa and globally, 61% and 79%, respectively, of those aware of their HIV-1 positive status were on HAART in 2017 (UNAIDS, 2018).

1.4.1 *Pre-exposure prophylaxis*

Pre-exposure prophylaxis (PrEP) involves the use of ARVs to prevent HIV-1 acquisition in high-risk individuals (reviewed by Riddell *et al.*, 2018). In 2012, PrEP consisting of a fixed dose combination of TDF and FTC was approved for use in individuals at high risk of HIV-1 infection (Riddell *et al.*, 2018). The most clinically advanced and efficacious microbicides under development incorporate the ARVs TFV/TDF, dapivirine (DPV) and maraviroc (MVC), either alone or in combination (Thurman *et al.*, 2013).

1.4.2 *Tenofovir disoproxil fumarate*

TDF is a nucleotide analog reverse transcriptase inhibitor (NRTI), indicated for use in the prevention and treatment of HIV-1 infection (Gallant and Deresinski, 2003). It is also used to treat chronic hepatitis B virus infection and has antiviral activity against HIV-2 (a distinct lineage of HIV) (Gallant and Deresinski, 2003). TDF is a prodrug of TFV (Grim and Romanelli, 2003; Gallant and Deresinski, 2003), and given its relatively benign safety profile and high efficacy in both treatment-naïve and experienced patients, is the most commonly administered NRTI (Arts and Hazuda, 2012). TDF requires initial diester hydrolysis for conversion to TFV, which is then converted to its active form, tenofovir diphosphate (TFV-DP), through two consecutive phosphorylation steps (Balzarini *et al.*, 1991). TFV-DP competes with intracellular deoxyadenosine

triphosphate for incorporation into the nascent complementary (c)DNA strand by HIV-1 reverse transcriptase. Seeing as TFV-DP lacks a 3' hydroxyl group, its incorporation into the DNA strand results in the termination of further synthesis (De Clercq, 2007; Kelly and Shattock, 2011).

TFV was the first ARV to demonstrate effectiveness and safety for use as a microbicide (Kelly and Shattock, 2011). The CAPRISA 004 trial was a two-arm, double-blind, randomized, placebo-controlled study that assessed the effectiveness of a 1% TFV vaginal gel in South African women and concluded the microbicide reduced the incidence of HIV-1 acquisition by an estimated 39% (54% in high adherers) (Abdool Karim *et al.*, 2010). However, the Vaginal and Oral Interventions to Control the Epidemic (VOICE) and Follow-on African Consortium for Tenofovir Studies (FACTS) 001 confirmatory trials failed to demonstrate the effectiveness of the TFV gel at preventing HIV-1 infection, which was ascribed to low adherence by participants (Marrazzo *et al.*, 2015; Delany-Moretlwe *et al.*, 2018). TDF and TFV, unlike many other ARVs, are not metabolised by the cytochrome P450 family of drug-metabolizing enzymes; TFV is excreted unchanged, renally (Kearney *et al.*, 2004). TDF peak serum and cervical tissue concentrations and *in vitro* IC_{50} in peripheral blood mononuclear cells (PBMCs) are detailed in **Table 1.2**, and the chemical structure of TFV and TDF are shown in **Fig. 1.2**.

1.4.3 *Dapivirine*

DPV is a non-nucleoside reverse transcriptase inhibitor (NNRTI); a lipophilic compound which specifically inhibits the replication of HIV-1 (but not other retroviruses, including HIV-2) by binding to a specific allosteric site of HIV-1 reverse transcriptase (Ding *et al.*, 1995; De Clercq 2004). Binding of NNRTIs to this allosteric site, which is thought to have a flexible structure that allows for effective DNA polymerization, prevents its mobility and therefore cDNA synthesis (Ding *et al.*, 1995).

The International Partnership for Microbicides (IPM) is evaluating the use of DPV as a microbicide in the form of a monthly intravaginal ring (IVR), a 90-day MPT IVR combining DPV and LNG, as well as a daily intravaginal gel (Baeton *et al.*, 2016, 2018; Nel *et al.*, 2018). A Study to Prevent Infection with a Ring for Extended Use (ASPIRE), was a randomized, double blind, placebo-controlled trial of a monthly DPV IVR in Sub-Saharan African women, that concluded that the IVR reduced the overall incidence of HIV infection by 27% (Baeten *et al.*, 2016). ASPIRE found no adverse effects in women using the IVR; its safety was equivalent to that of the placebo

IVR utilized in the study (Baeten *et al.*, 2016). A similar study, The Ring Study, found that HIV-1 acquisition in users of a monthly DPV ring was 31% lower than in users of a placebo IVR (Nel *et al.*, 2016). The results of ASPIRE and The Ring Study represent the first time two separate Phase III trials have shown a microbicide to be clinically efficacious. Interim results from the HIV Open Label Prevention Extension (HOPE) and Dapivirine Ring Access and Monitoring (DREAM) studies (open-label extension trials of ASPIRE and The Ring Study, respectively) demonstrated HIV-1 incidence at half of the expected rate in DPV IVR users (Baeton *et al.*, 2018; Nel *et al.*, 2018). *In vitro*, DPV has been shown to be metabolised by several members of the cytochrome P450 family of enzymes (To *et al.*, 2013). DPV peak serum and cervical tissue concentrations and *in vitro* IC_{50} in PBMCs are reported in **Table 1.2**, and DPVs chemical structure is shown in **Fig. 1.2**.

1.4.4 *Maraviroc*

Maraviroc (MVC) is a potent, selective, reversible antagonist of the CCR5 chemokine receptor (Lieberman-Blum *et al.*, 2008). Its antiretroviral effects arise from binding competitively to the CCR5 receptor, thereby preventing R5 tropic HIV-1gp120 from binding to its coreceptor. As a result, the conformational changes required for the activation of gp41 do not occur, the virus is unable to gain entry into the cell and productive infection is not initiated (Dorr *et al.*, 2005). The IPM conducted a Phase I clinical study assessing the pharmacokinetics and pharmacodynamics of IVRs containing DPV only, MVC only or DPV and MVC in combination (Chen *et al.*, 2015). It concluded that the levels of DPV, but not MVC in cervical tissue of IVR users were sufficient to prevent HIV-1 viral replication (Chen *et al.*, 2015). Likewise, Fletcher *et al.* showed MVC (at concentrations up to 1 μM) had no significant anti-HIV-1 activity in ectocervical tissue (Fletcher *et al.*, 2016). MVC is metabolized extensively by members of the cytochrome P450 family of drug-metabolizing enzymes (Tseng *et al.*, 2018). MVC peak serum concentrations and *in vitro* IC_{50} are reported in **Table 1.2**, and MVCs chemical structure is shown in **Fig.1.2.**

Table 1.2: **TDF, DPV and MVC peak serum concentrations, cervical tissue concentrations and *in vitro* IC_{50} in PBMCs.**

ARV	Mode of administration	Peak serum concentration (nM)	Cervical tissue concentration (µM)	*In vitro* IC_{50}♦ in PBMCs (nM)
TDF	365 mg TDF ring	1.2–5.2[1]	9–30[1]	5–10[3]
	300 mg TDF/200 mg FTC oral PrEP (once daily)	77–87[2]	79[2]	
	300 mg TDF oral pills (HAART) (multiple daily doses)	1100–1140[3]	-	
DPV	25 mg DPV ring	0.7–0.89[4]	2–21[5-7]	2.4[8]
MVC	300 mg oral (twice daily)•	1170[9]	-	1.2[10]

References: [1] Keller *et al.*, 2016; [2] Hendrix *et al.*, 2016; [3] Fung *et al.*, 2002; [4] Chen *et al.*, 2015; [5] Romano *et al.*, 2009; [6] Nel *et al.*, 2009; [7]; Nel *et al.*, 2016; [8] Fletcher *et al.*, 2009; [9] Abel *et al.*, 2008; [10] Dorr *et al.*, 2005. Symbols; [•] United States Food and Drug Administration general recommended dose in adults; [♦] IC_{50} is defined as the concentration of drug required to inhibit HIV-1 replication by 50%.

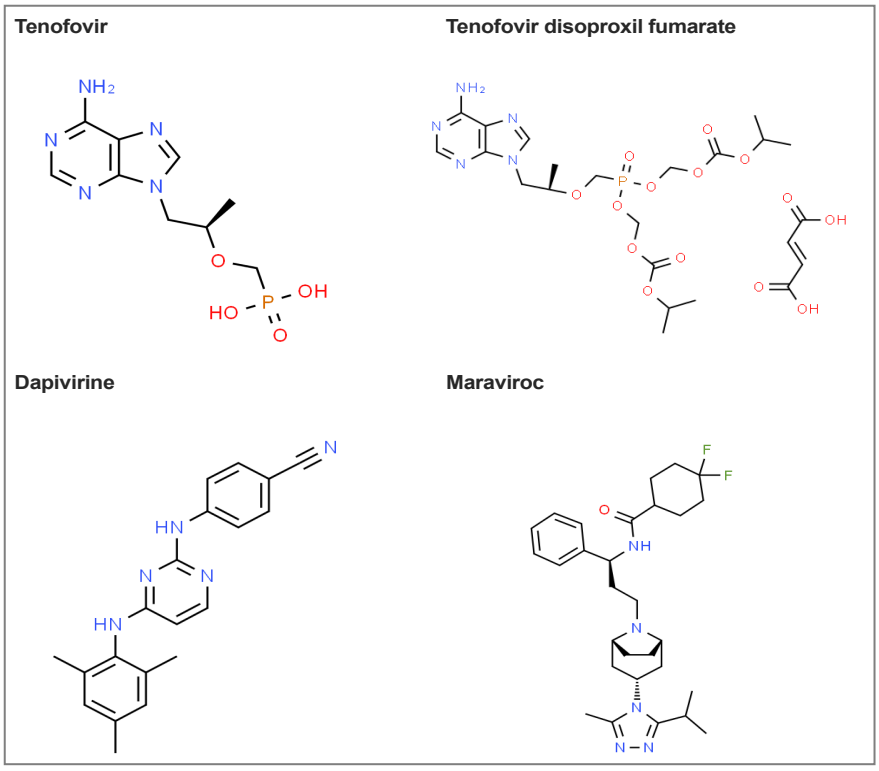

Figure 1.2: **Chemical structures of TFV, TDF, DPV and MVC.** TDF requires initial diester hydrolysis for conversion to TFV, which is then is converted to its active form TFV-DP, through two consecutive phosphorylation steps. DPV binds to a specific allosteric site of HIV-1 reverse transcriptase, thus inhibiting its action, MVC is a reversible antagonist of the CCR5 chemokine receptor. Chemical structures were obtained from ChemSpider at http://www.chemspider.com.

1.5 *Immunomodulatory and adverse effects of ARVs*

For the development of successful PrEP strategies, it is necessary their immunomodulatory effects be characterised. TDF, DPV and MVC have been shown to have immunomodulatory effects, independent of their antiviral effects.

I.5.1 *TFV modulates cytokine production*

TFV appears to selectively modulate cytokine production in PBMCs (Melchjorsen *et al.*, 2011). In monocytes, following stimulation with TLR ligands, tumor necrosis factor (TNF)-α or live pathogens, TFV decreases IL-8 and MIP-1α cytokine secretion (Melchjorsen *et al.*, 2011). TFV similarly decreases MIP-1α protein levels, as well as anti-inflammatory IL-10 and increases proinflammatory IL-12 protein and mRNA expression levels in human PBMCS (Melchjorsen *et al.*, 2011). Melchjorsen *et al.* also found no effect of TDF on NF-κB or mitogen activated protein kinase (MAPK) signaling (Melchjorsen *et al.*, 2011). Another group showed human PBMCs exposed to TFV had significantly increased secretion of regulated upon activation, normal T cell expressed and secreted (RANTES) and MIP-1α, but not MIP-1β or IL-6 (Zidek *et al.*, 2007).

Hladik *et al.* showed a reduced glycerin rectal 1% TFV gel altered the expression of a broad range of genes in rectal biopsies, including innate immune function genes and genes involved in DNA damage repair (Hladik *et al.*, 2015). In this study, approximately 500 and 100 genes were downregulated and upregulated, respectively. Notably, several transcription factors, including nuclear factor of activated T cells (NFAT) and cyclic AMP response element-binding protein (CREB)1 were amongst the genes downregulated (Hladik *et al.*, 2015). In addition, application of the TFV gel induced proinflammatory chemokine C-C motif ligand (CCL)19 and CCL21 gene expression and suppressed anti-inflammatory IL-10 and TGF-β expression (Hladik *et al.*, 2015). The authors postulated IL-10 suppression by the TFV gel may have been mediated by downregulating the expression of (CREB)1 and its coactivator CREB-binding protein (CBP) (Hladik *et al.*, 2015). In another study, TFV was shown to increase proinflammatory IL-8 and TNF-α secretion in endometrial and ectocervical primary epithelial cells (Biswas *et al.*, 2014). TNF-α has been shown to disrupt tight junction proteins between intestinal and genital ECs *in vitro*, and in this way impair barrier function (Nazli *et al.*, 2010). Similarly, in endometrial and cervical ECs and fibroblasts, TFV has been shown to compromise tight junctions, and inhibit wound closure *in vitro* (Rodriguez-Garcia *et al.*, 2017). A possible link between TFV-induced decreases in mucosal

permeability, delayed wound healing and increased risk of HIV-1 infection deserves further investigation. The mechanism through which TFV modulates cytokine and chemokine production in the *in vitro* studies reported here remains undetermined. Whether these effects are mediated via the GR, which broadly regulates immunomodulatory function, including cytokine and chemokine production (see **Section 1.6**) has not been investigated directly. Moreover, whether TDF has similar immunomodulatory effects *in vivo*, and if so, the implications on HIV-1 susceptibility in women using the ARV prophylactically, has not been established.

1.5.2 *TDF use is associated with osteoporosis and bone fractures*

HIV-1 infected individuals have higher incidences of osteoporosis and bone fractures compared to the general population (Brown and Qaqish, 2006). Multiple factors, including the effect of inflammatory cytokines (Steeve *et al.*, 2004), HIV-1 proteins (Fakruddin and Laurence, 2005), as well as HAART on bone are thought to be responsible (Brown *et al.*, 2009). Although studies have shown a loss of BMD following HAART initiation, irrespective of regimen, TDF-containing regimens are associated with greater losses in BMD (Brown *et al.*, 2009). Numerous clinical, animal and *in vitro* studies support the association of TDF use with decreases in BMD and increases in markers of bone turnover. A recent observational cohort study found TDF treatment, over a period of 5 years or more, significantly increased the risk of osteoporosis related bone fractures in young Japanese men (Komatsu *et al.*, 2018). In human osteoblast primary cell cultures, TDF has been shown to dose-dependently decrease both mineralization, as well as the expression of collagen, the main component of bone matrix (Barbieri *et al.*, 2018). In another study, microarray analysis of TDF-treated primary murine osteoblasts identified 79 transcripts with significantly altered expression profiles in TDF- vs. non-TDF-treated cultures (Grigsby *et al.*, 2010). Notably, the expression of genes involved in cell signaling transduction pathways, amino acid metabolism and biosynthesis were downregulated, the effects of which are likely to be reductions in osteoblast growth and differentiation (Esposito *et al.*, 2015). Significant changes in genes involved in the Wingless/Integrated (Wnt), TGF-β, Hedgehog and MAPK signaling pathways were identified (Grigsby *et al.*, 2010). Whether the effects of TDF on bone are mediated by the GR and GCs, which regulate bone cell differentiation, development and function (see **Section 1.6.8**) has not been determined.

1.5.3 *Immunomodulatory effects of DPV*

The limited studies performed with DPV, which has not currently been approved for clinical use, have shown some proinflammatory effects. In endometrial cancer cells, at relatively high concentrations (10 μM and 100 μM), DPV has been shown to induce proinflammatory IL-8 secretion (Gali *et al.*, 2010). Similarly, in VK2/E6E7, CaSki and Caco-2 cells 100 μM DPV has been shown to induce apparent increases in IL-6 and IL-8 secretion (des Neves *et al.*, 2013). Conversely, another study by des Neves *et al.* found DPV-loaded nanoparticles did not increase IL-8, IL-6, MIP-3α, IL-1α or IL-1ß expression in a murine model (des Neves *et al.*, 2014). Adverse systemic effects resulting from the use of DPV have not been reported; in the DPV IVR trials conducted to date, the adverse effects reported have mostly been as a result of IVR fitment and immunomodulatory effects have not been investigated (Nel *et al.*, 2009, 2014). The few *in vitro* studies that have assessed the immunomodulatory effects of DPV have not determined the mechanism through which DPV induces cytokine and chemokine production in some cells. Whether DPV in some way alters GR activity has not been investigated. It is unclear whether DPV has similar immunomodulatory effects *in vivo*, and if so, the implications on HIV-1 susceptibility in women using the ARV prophylactically.

1.5.4 *Immunomodulatory effects of MVC*

MVC is currently the only approved ARV that targets a human protein (Lieberman-Blum *et al.*, 2008). Numerous CCR5 ligands in tandem stimulate T cell activation, the initiation of immune responses and migration of immune cells to sites of infection (reviewed by Lederman *et al.*, 2006). CCR5 also promotes IL-2-dependent events, NFAT activation, lymphocyte recruitment and proliferation (Gaitan *et al.*, 2010). Although the immunological effects of CCR5 inhibition are not well established, MVC has been demonstrated to induce proinflammatory gene expression in several studies. A recent study showed MVC-dependent NF-κB (but not AP-1 or NFAT) activation, and increases in the expression of NF-κB dependent proinflammatory gene expression (IL-6, IL-10 and TNF-α) both in resting CD4+ T cells from HIV-1 positive patients taking MVC, and in a CCR5 expressing cervical cell line (Madrid-Elena *et al.*, 2018). The mechanism was determined to be dependent on MVC binding CCR5 and subsequent induction of downstream signalling pathways (Madrid-Elena *et al.*, 2018). MVC induced NF-κB activation was associated with the reversal of HIV-1 latency in infected CD4+ T cells in the same study (Madrid-Elena *et al.*, 2018). A recent randomized, placebo-controlled study in HIV-1 positive individuals found

MVC intensification caused increases in rectal and peripheral blood T cell activation, increases in MIP-1ß, as well as increases in neutrophil levels and monocyte activation (Hunt *et al.*, 2018). In contrast, other studies have shown decreases in T cell activation upon initiation of HAART regimens with MVC (Wilkin *et al.*, 2012). An *in vitro* study found MVC treatment tended to decrease expression of markers of immune activation in both CD4+ and CD8+ T cells, and dose-dependently inhibited chemokine-induced T cell migration (Arberas *et al.*, 2013). Whether MVCs immunomodulatory effects contribute to its failure to suppress HIV-1 infection in mucosal tissues when used as PrEP has not been determined.

1.5.5 *Interactions between antiretroviral drugs and progestins*

Most ARVs used in HAART or PrEP have limited effects on the contraceptive efficacy of HCs (reviewed extensively by Nanda *et al.*, 2017). The NNRTI efavirenz (EFV) is an exception, and has been reported to reduce serum concentrations of both LNG and etonorgestrel (ETG, another progestin used in subdermal implants) (Nanda *et al.* 2017). In one study, HAART with EFV reduced ETG levels by up to 70% in women using the progestin subdermal implant, compared to HAART without EFV (Vieira *et al.*, 2014). Similarly, in women using COCs with LNG, EFV alone, or in combination with other ARVs reduced progestin levels by approximately 60% (Sevinsky *et al.*, 2011; Landolt *et al.*, 2013). LNG levels were reduced by a similar extent in emergency contraceptive pill users after using EFV (Carten *et al.*, 2012). The mechanism by which EFV reduces ETG and LNG serum concentrations is thought to be through EFV-induced expression of the enzyme cytochrome P450 3A in hepatocytes, which metabolizes these progestins (Hariparsad *et al.*, 2004; Korhonen *et al.*, 2005) Studies have shown HAART with EFV does not impact MPA serum concentrations or ovulation in the weeks following a 150 mg MPA injection (Cohn *et al.*, 2007; Nanda *et al.*, 2008). It should be noted however that changes in HC levels do not necessarily translate to reduced contraceptive efficacy; progestin serum concentrations vary greatly between individuals, suggesting prescribed doses are far above what is required for contraceptive efficacy (see **Table 1.1**) (Hapgood et al., 2018). The effects of progestins on EFV pharmacokinetics is less clear, although it appears progestins differentially influence EFV serum concentrations (Nanda *et al.* 2017). While one study has shown that compared to historical controls, COCs with the progestin desogestrel reduced EFV serum concentrations in HIV-1 positive women (Landolt *et al.*, 2014), other studies have shown no effect of COCs with the progestin norgestimate, or DMPA on EFV levels (Burger *et al.*, 2006; Cohn *et al.,* 2007; Sevinsky *et al.*, 2011).

TDF-FTC oral PrEP does not affect the contraceptive efficacy of several HCs (Nanda *et al.*, 2017). Co-administration of TDF and COCs with norgestimate, or implants with LNG has been shown to result in no significant drug-drug interactions, or effects on contraceptive efficacy (Kearney and Mathias, 2009; Todd *et al.*, 2015) . Retrospective analysis of two TDF-FTC PrEP trials found TDF-FTC PrEP had no effect on pregnancy rates among users of implants, COCs or MPA injectables (Murnane *et al.*, 2014; Callahan *et al.*, 2015). MVC has been shown not to alter COC LNG levels, or the pharmacokinetics of other ARVs (Abel, *et al.*, 2008). Abel *et al.* also reported MPA use did not alter MVC serum levels in women (Abel, *et al.*, 2008). When assessing factors such as CD4+ T cell counts, plasma HIV-1 viral loads and death, studies have found no effect of MPA injectables, LNG implants or COCs on the effectiveness of HAART with NNRTIs or protease inhibitors (Watts *et al.*, 2008; Nanda *et al.*, 2017). Furthermore, concurrent use of HAART and progestins does not appear to alter adverse side effects (Nanda *et al.*, 2017). Both TDF and MPA use are associated with BMD loss. However, in women using TDF-FTC PrEP and MPA concurrently, no significant alterations in BMD were reported (Kasonde *et al.*, 2014).

A study reported DMPA did not affect the efficacy of TDF-FTC PrEP in heterosexual Kenyan and Ugandan HIV-1 serodiscordant couples (Heffron *et al.*, 2014). In contrast, one *in vitro* study reported MPA suppressed the protective effect of TFV on HIV-1 infection and lowered TFV-DP concentrations in blood CD4+ T cells, unlike LNG, P_4 or another injectable HC, norethisterone (Shen *et al.*, 2017). Shen *et al.* also showed MPA supressed tenofovir alafenamide (TAF, another prodrug of TFV) inhibition of HIV-1 infection and lowered TFV-DP concentrations in endometrial CD4+ T cells (Shen *et al.*, 2017). A study assessing if pregnancy reduces TFV and TFV-DP concentrations in Ugandan and Kenyan women using TDF-FTC oral PrEP concluded TFV and TFV-DP serum concentrations were 45–58 % lower during pregnancy, although it was not clear if this undermined the effectiveness of PrEP in these women (Pyra *et al.*, 2018). Other studies have reported higher TFV clearance during pregnancy, compared to periods women were not pregnant (Benaboud *et al.*, 2012; Best *et al.*, 2015). However, viremic suppression in these studies was found to be similar between pregnant and non-pregnant women using TFV (Benaboud *et al.*, 2012; Best *et al.*, 2015).

1.6 *The glucocorticoid receptor*

The GR is a ubiquitous intracellular steroid receptor, and a member of the nuclear receptor superfamily (reviewed extensively by Lu *et al.*, 2006). Nuclear steroid receptors share a similar structural organization comprised of a central highly conserved zinc-finger DNA-binding domain (DBD), a less conserved carboxy-terminal ligand-binding domain (LBD) and dissimilar amino-terminal domains (NTD) (reviewed by Weikum *et al.*, 2018). Like other steroid receptors, the GR is a ligand-activated transcription factor (Yudt and Cidlowski, 2016). Glucocorticoids (GCs) acting via the GR, regulate virtually all aspects of physiology, including stress responses, metabolism, immune function, growth, reproduction and homeostasis (reviewed by Baschant and Tuckermann, 2010). In response to physiological and psychological stressors, cortisol (CORT), an endogenous GC secreted from the adrenal cortex in response to adrenocorticotropic hormone (ACTH), is released (Padgett and Glaser, 2003; Schäcke *et al.* 2016). Through regulating cellular trafficking, proliferation, cytolytic activity, differentiation, effector function, cytokine and chemokine secretion and antibody production in leukocytes and lymphocytes, CORT exerts potent immunosuppressive and anti-inflammatory effects in the context of infection, increased immune activation or inflammation (Padgett and Glaser, 2003; Lu *et al.*, 2006).

1.6.1 *GR mechanism of action*

A single gene, produces two GR isoforms; GRα, the predominant GR isoform and GRβ, an alternative isoform present in the nucleus of some cells (Hollenberg *et al.*, 1985). Unlike GRα, GRβ is unable to bind to known GCs, and does not transcribe the majority of GC-responsive genes (de Castro *et al.*, 1996). Although they modulate each other's transcriptional activity on a few genes, GRβ transcribes a mostly distinct array of genes from GRα (Kino *et al.*, 2009). GRβis a dominant negative isoform, and therefore an inhibitor of GC action (Bamberger *et al.*, 1995; Zhou and Cidlowski, 2005). Throughout this text, GR refers to GRα.

Prior to ligand binding, the GR is typically localized in the cytoplasm as part of a multiprotein complex which includes heat shock proteins (Hsp)90, Hsp70, Hsp40 and Hsp23, immunophilins, phosphatases and Hsp90 co-chaperone p23, and is held in an inactive but receptive conformation (Ramamoorthy and Cidlowski, 2013). In the serum, CORT is predominantly bound to corticosteroid-binding globulin, the levels of which determine the proportion of serum free, bioavailable CORT (Gardill *et al.*, 2012). Upon ligand binding, ligands induce ligand-specific

conformations both in the LBD, and other domains of the GR, which determine co-regulator association, as well as GR binding to its recognition elements (Gass *et al.*, 1998, Ronacher *et al.*, 2009). Two modes of GR transcriptional regulation, termed transactivation and transrepression, are best characterized.

1.6.2 Transactivation and transrepression

Typically, the GR binds to glucocorticoid response elements (GREs) in the promoters of target genes as a dimer (Zhou and Cidlowski, 2005). GREs are palindromic nucleotide sequences, with a consensus sequence of GCTACAnnnTCTTCT (Nordeen *et al.*, 1990). Variability in the sequence allows for variant GR/DNA binding, and consequently, varying gene-specific GR responses (Kumar *et al.*, 1999). The GR is thought to act as a pioneering factor through recruiting chromatin remodelling complexes, and cofactors necessary for transcription initiation, in this way allowing access for basal transcription machinery to the promoters of GC-responsive genes (reviewed. By Burd and Archer, 2013). Through transactivation, the GR regulates anti-inflammatory genes (for example MAPK phosphatase-1 (MKP-1) (Lasa *et al.*, 2002), inhibitor of kappa B alpha (IκB-α) and glucocorticoid induced lysine zipper (GILZ) (Deroo and Archer, 2001).

Transrepression typically involves the GR tethering to pro-inflammatory transcription factors like NF-κB, AP-1 and NFAT, and downregulating the expression of pro-inflammatory genes, for example IL-6 and IL-8 (De Bosscher *et al.* 2000; Nissen and Yamamoto, 2000). This is achieved through the recruitment of chromatin remodelling complexes and co-repressors (Chinenov *et al.* 2013). However, transrepression mechanisms involving direct DNA-binding of the GR to negative GREs have been described (Ramamoorthy and Cidlowski, 2013). Transrepression and transactivation are depicted in **Fig. 1.3.**

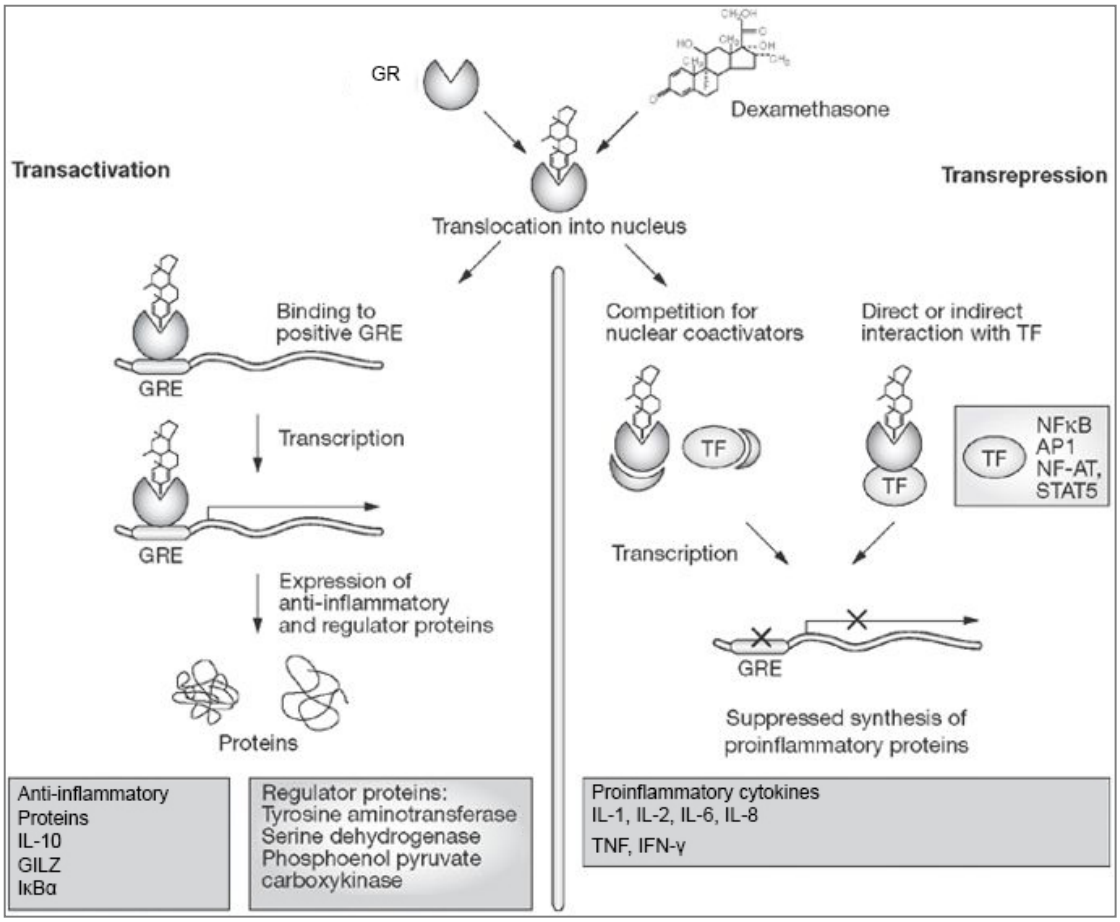

Figure 1.3: Genomic actions of glucocorticoids. Transactivation typically involves the GR binding to GREs in the promoters of target genes as a dimer, recruiting chromatin remodelling complexes and transcription machinery and activating transcription of anti-inflammatory and regulatory genes. Transrepression typically involves the GR tethering to pro-inflammatory transcription factors like NF-κB AP-1, and downregulating the expression of pro-inflammatory genes. Figure adapted from (Stahn and Buttgereit, 2008).

In addition to the classical genomic actions described above, GCs have rapid non-genomic actions (Buttgereit and Scheffold, 2002; Stahn and Buttgereit, 2008). At high concentrations, GCs can intercalate into membranes, and affect their permeability (Buttgereit and Scheffold, 2002; Stahn and Buttgereit, 2008). In this way, GCs can decrease adenosine triphosphate (ATP) synthesis, as well as calcium and sodium shuttling across the membrane, which are essential for the actions of immune cells, including phagocytosis, cytokine and chemokine production, migration and antigen processing and presentation (Buttgereit and Scheffold, 2002; Stahn and Buttgereit, 2008). Members of the multiprotein complex to which the GR is bound, which are released upon ligand-binding, for example steroid receptor co-activator (SRC) and MAPK1 are thought to mediate some of the rapid non-genomic effects of GCs (Croxtall *et al.*, 2000; Buttgereit and Scheffold, 2002). Membrane-bound GR has been identified in human PBMCs (Bartholome *et al.*, 2004), and is thought to be involved in pro-apoptotic, immunomodulatory and metabolic effects which prime

the actions of the cytosolic GR (Vernocchi *et al.*, 2013). Upon GCs binding to membrane-bound GR, signal transduction cascades are induced in the cell via the activation of kinases, including p38 MAPK, which mediate GC rapid non-genomic effects (Strehl and Buttgereit, 2014). In addition, activation of signal transduction pathways may result in changes in gene expression (Strehl and Buttgereit, 2014).

It should be noted that apart from control at the level of transcription initiation, mRNA posttranscriptional processing, mRNA trafficking from the nucleus to the cytoplasm and control at the level of translation contribute to the regulation of GR-mediated gene expression (Kumar. et al., 2014). Post-transcriptional regulation often involves alterations to the rate of mRNA decay, mediated through deadenylation-dependent pathways or endonucelases (reviewed by Garneau et al., 2007). mRNA decay has been shown to account for up to 50% of changes in mRNA levels in some responses (Garneau et al., 2007). In addition, alterations to mRNAs 5' terminal cap or 3' poly(A) tail allow RNA-binding proteins to repress or activate translation (Baker and Coller, 2009). Post-transcriptional regulation may also involve micro-RNAs, which inhibit translation initiation or elongation, as well as post translational modifications of ribosomes and translational apparatus (Baker and Coller, 2009).

1.6.3 *The chromatin landscape determines cell-specific GC responses*

Although the GR is ubiquitously expressed, it regulates gene expression in a cell-specific manner (Burd and Archer, 2013). It has become apparent the model of GR transactivation described above is insufficient, and does not encompass the diversity of genomic interactions and mechanisms which determine GR specificity (Burd and Archer, 2013). The majority of GR binding sites, in fact, occur far from classical promoter sequences (Hakim *et al.*, 2009; Reddy *et al.*, 2009). The GR is now understood to mediate its effects through interactions with distal enhancer elements, many kB from the promoters of target genes, through tethering and alternate recognition motifs (Hakim *et al.*, 2009; Reddy *et al.*, 2009). The genome is believed to be organized in a manner which allows linkage of these distal enhancer sites to genes by altering chromatin dynamics, DNA topology and specialized chromatin structures (Hager and Varticovski, 2012; Burd and Archer, 2013). Contrary to the views of the classical model, the GR has been shown to preferentially bind to deoxyribonuclease (DNAse) I hypersensitive sites, where chromatin exists in an accessible state (John *et al.*, 2008). Therefore, variation in the distribution of DNAse I hypersensitive sites in chromatin further allows the programming of cell-specific GR responses (Burd and Archer, 2013).

1.6.4 *Co-regulators modulate GR activity*

In excess of 400 co-regulators, which are intrinsically involved in the regulation of all aspects of physiology, have been described (Lonard and O'Malley, 2012). These are typically classified as either co-activators, which classically interact with ligand-bound receptor and assist in the activation of gene expression, or co-repressors, which typically bind to unliganded receptor to repress gene expression (Lonard and O'Malley, 2012). Co-activator and co-repressor function, which are gene-promoter dependent, can be altered through post-translational modifications, further fine-tuning transcriptional control (Chinenov *et al.*, 2012; Lonard and O'Malley, 2012). GR interactions with the steroid receptor co-activator (SRC) family of transcription factors, SRC-1, SRC-2 (also known as GRIP-1) and SRC-3 are well characterized (Clarisse *et al.*, 2017). These co-activators participate in the formation of multiprotein complexes, which include chromatin remodelling enzymes (Lee and Stallcup, 2017), and are crucial for the recruitment of basal transcription machinery and the ordered, dynamic process of transcription initiation (Lonard and O'Malley, 2012). Co-activators which commonly interact with the GR in multiple cells types include SRC-1/2/3, peroxisome proliferator-activated receptor gamma co-activator 1-alpha, receptor-interacting protein 140 and DAX-1 (Clarisse *et al.*, 2017).

1.6.5 *GR phosphorylation*

The GR is regulated through direct phosphorylation by serine/threonine kinases (reviewed extensively by Hapgood *et al.*, 2016; Kino, 2018). Five serine (Ser) residues in the GR NTD– Ser124, Ser203, Ser211, Ser226 and Ser404 have been identified as serine/threonine kinase targets for phosphorylation (**Fig. 1.4**) (Hapgood *et al.*, 2016, Kino, 2018). Phosphorylation of the GR at Ser203 by cyclin dependent kinase (CDK) 1 and 5 has been shown to occur in the absence of ligand, and is associated with cytoplasmic (Blind and Garabedian, 2008) and perinuclear (Chen *et al.*, 2008) retention of the GR. GR phosphorylation at Ser203 therefore restricts nuclear import, and subsequent GR recruitment to the promoters of target genes (Blind and Garabedian, 2008). Phosphorylation of the GR at Ser134 by Ak strain transforming (AKT)1 inhibits GR translocation to the nucleus and suppresses GC-induced gene expression (Piovan *et al.*, 2013).

Phosphorylation of the GR at Ser211, which occurs upon ligand binding, has been shown to be associated with GR recruitment to the promoter of target genes (Blind and Garabedian, 2008), and achieving a maximal GR transactivational response (Chen *et al.,* 2008, Avenant *et al.,* 2010a).

CDK5 (Kino *et al.*, 2007) and c-Jun N-terminal kinase (JNK) (Itoh *et al.*, 2002) have been shown to phosphorylate the GR at Ser211. Adenosine 5' monophosphate-activated protein kinase (AMPK) activates the GR indirectly, through phosphorylating MAPK which in turn phosphorylates Ser211 (Nader *et al.*, 2010). Ser226 on the GR is hyperphosphorylated in the presence of ligand by CDK5, and has been shown to modulate cofactor recruitment to DNA-bound GR (Kino *et al.*, 2007). Phosphorylation of the GR at Ser226 has been shown to enhance nuclear export (Itoh *et al.*, 2002) and is thus associated with blunting GR responses (Kino *et al.*, 2007). A Ser226 to alanine mutation is associated with increased GR transactivational responses (Avenant *et al.* 2010b). The GR is phosphorylated at Ser404 by glycogen synthase kinase (GSK)3β, which has been shown to alter cofactor recruitment to the GR and GC-dependent NF-κB transrepression, thus attenuating pro-inflammatory responses (Galliher-Beckley *et al.*, 2008).

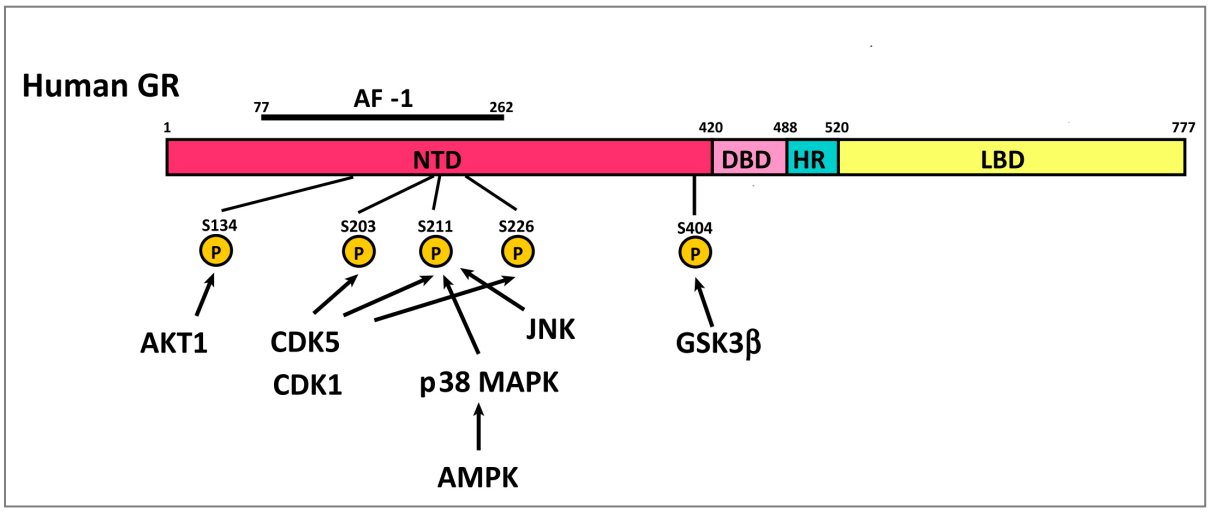

Figure 1.4: **The GR is phosphorylated at Ser124, Ser203, Ser211, Ser226 and Ser404 in the NTD by serine threonine kinases**. Phosphorylation at these residues modulate GR transactivation, transrepression, co-regulator recruitment and nuclear translocation. NTD; amino-terminal domain, DBD; DNA binding region, HR; hinge region, LBD; ligand binding domain. Figure adapted from (Kino, 2018).

1.6.6 *GC-independent modulation of GR activity*

The GR can be activated in the absence of GCs, by a variety of factors, through cross-talk between signal transduction pathways (Hapgood *et al.*, 2016). Gonadotropin releasing hormone (GnRH) has been shown to induce GR transactivation of a GRE-reporter gene in a mouse gonadotrope LβT2 cell line, through a mechanism involving GnRH receptor activation, and protein-kinase-C- and MAPK-dependent phosphorylation of the GR at a residue equivalent to Ser226 on the human GR (Kotitschke *et al.*, 2009). TNF has been shown to activate the endogenous GR in the absence of GCs, and to repress IL-6 expression in the End/E6E7 human

endocervical cell line (Verhoog *et al.*, 2011). Using ChIP, Verhoog *et al.* determined that TNF enhanced GR and GRIP-1 recruitment to the IL-6 promoter, as well as GR phosphorylation at Ser226, in the absence of GCs (Verhoog *et al.*, 2011). Alternatively, non-GC ligands can modulate GR activity through synergism, which occurs when two factors, which individually induce a response, induce a response greater than the sum of their individual responses when in combination (Chou, 2006). For example, LPS and GCs have been shown to inhibit inflammation in macrophages by synergistically upregulating sphingosine kinase 1 expression, possibly mediated by their synergistic effect on GR recruitment to the promoters of genes (Vettorazzi *et al.*, 2015). Additionally, the coactivation of the GR and the transcription factors NF-κB and AP-1 is known to enhance proinflammatory responses (reviewed by Busillo and Cidlowski, 2013).

The GR's activity can also be primed/sensitized by growth factors, cytokines and β2-adrenoreceptor agonists, which on their own do not activate the GR, but rather, increase transcriptional responses in the presence of GCs (Hapgood *et al.*, 2016). ln cultured human airway smooth muscle cells, Hu *et al.* showed that the proinflammatory cytokine IL-13, in the absence of GC, did not induce transcription of a synthetic GRE-reporter gene, but in combination with GC, the reporter gene was transcribed to a greater extent than with GC alone (Hu *et al.*, 2013). IL-13 also potentiated GC-induced phosphorylation at Ser211 and GC-induced nuclear translocation (Hu *et al.*, 2013). Moreover, IL-13, in the absence of GC, induced phosphorylation of the GR at Ser203 and Ser211, in a process mediated by extracellular signal regulated kinase (ERK)-1/2 and JNK, and allowed the unliganded GR to bind to the mediator complex subunit-14 co-activator (Hu *et al.*, 2013). Long-acting β(2)-adrenoreceptor agonists (LABAs) have been shown to potentiate GC effects, and are used to enhance the clinical efficacy of GCs used therapeutically (reviewed by Newton and Giembycz, 2016). For example, the LABA salmeterol has been shown to increase GC-induced transactivation of the MKP-1 gene, and a GRE-reporter gene in primary macrophages, presumably through its enhancement of GC-induced nuclear translocation and DNA-binding (Haque *et al.*, 2013). Another LABA, formoterol, and has been shown to enhance GR nuclear translocation in PBMCs, as well as TNF-induced IL-8 production, through a mechanism involving a decrease in total GR phosphorylation, which was associated with decreased activation of the kinases JNK and p38-MAPK, which are known to enhance nuclear export of the unliganded GR (Mercado *et al.*, 2011). Moreover, salmeterol and formoterol have been shown to increase MKP-1 mRNA and protein expression in a manner involving the cAMP-PKA pathway in primary airway smooth muscle cells, concomitant with increasing GR nuclear

translocation and DNA-binding, as well as increasing GC-induced IL-6 and IL-8 production (Manetsch *et al.*, 2013).

On the other hand, some factors desensitize GR signaling, resulting in decreased responses to GC (Hapgood *et al.*, 2016). GC-independent phosphorylation of the GR at Ser134, mediated by p38 MAPK, and induced by a diverse range of cellular stressors, has been shown to gene-specifically desensitize response to GCs in U2OS cells (Galliher-Beckley *et al.*, 2011). In these cells, phosphorylation of the GR at Ser134 altered GR and 14-3-3 protein binding to the promoters of genes on chromatin (Galliher-Beckley *et al.*, 2011). Several cytokines have also been shown to desensitize GR signaling. For example IL-13, and the combination of IL-2 and IL-4, have been shown to reduce GC binding affinity to the GR, and ligand-bound GR nuclear translocation (Kam *et al.*, 1993; Spahn *et al.*, 1996). In A549 human epithelial cells, IL-1β has been shown to reduce the expression of some GC-induced genes, which was associated with IL-1β-induced reductions in cytoplasmic GR levels, the inhibition of GC-dependent GR nuclear translocation and phosphorylation of the unliganded GR at Ser203 and Ser211 (Escoll *et al.*, 2015). Taken together, ligand-independent modulation of GR activity appears to involve several diverse mechanisms, including site-specific alterations in GR phosphorylation, altered nuclear translocation of the unliganded GR, altered GR and cofactor recruitment or binding to the promoters of genes, as well as the altered activity or levels of signaling proteins, including kinases and cofactors, in the GR pathway.

1.6.7 *Potency, efficacy and biocharacter of a ligand*

Transcriptional responses induced by ligand-bound receptor are defined using dose-response curves. The shape of this curve allows the efficacy, potency and biocharacter of the ligand to be determined (Hapgood *et al.,* 2014a; Hapgood *et al.*, 2018). Efficacy refers to the maximal response elicited by a ligand, and potency is defined as the concentration of ligand required to achieve half maximal response (EC_{50}) (Hapgood *et al.,* 2014a, Hapgood *et al.*, 2018). The EC_{50} is dependent on the cell type, promoter and receptor concentrations and is variable between experiments (Robertson *et al.*, 2013; Hapgood *et al.*, 2014a). The efficacy is influenced by the affinity of the ligand for the receptor, receptor concentrations and the cell-specific relative concentrations of co-regulators (Ronacher *et al.*, 2009). Biocharacter is defined by the shape of the dose-response curve relative to a reference agonist, and a ligand may be an agonist (if it elicits the same maximal response as the endogenous ligand), partial agonist (if it elicits less than this maximal response) or antagonist (if it reduces the efficacy of an agonist) (Hapgood *et al.,* 2014a; Hapgood *et al.*, 2018).

1.6.8 *Therapeutic use of glucocorticoids*

GCs are widely used clinically as systemic and topical therapies for reducing inflammation and immune activation associated with a broad range of conditions including rheumatoid arthritis, allergies, inflammatory bowel syndrome, asthma, septic shock, pneumonia, adrenal insufficiency and skin conditions like eczema and dermatitis (Schäcke *et al.*, 2002). The majority of the therapeutic anti-inflammatory and immunosuppressive effects of GCs are mediated by transrepression (reviewed by Baschant and Tuckermann, 2010; Ramamoorthy and Cidlowski, 2013). In addition, GCs have anti-proliferative effects, through inducing cell cycle arrest, and their inhibition of cytokine production and DNA synthesis (Guichard *et al.*, 2015), as well as vasoconstrictive effects, mainly through inhibition of histamine and vasodilator synthesis (Yang and Zhang, 2004).

When used in medication, CORT is known as hydrocortisone (Garrod, 1958). Hydrocortisone is amongst the least potent GCs used therapeutically, is administered topically, orally or by injection, has both GC and mineralocorticoid activity and has a short duration of action (Garrod, 1958; Fardet and Fève, 2014, Nicolaides *et al.*, 2018). Reported endogenous and therapeutically administered CORT concentrations vary considerably; inconsistency in methods of administration, time post-administration at which serum concentrations are determined and experimental methodologies between studies are likely to account for many of the discrepancies between studies (see **Table 1.3**). Dexamethasone (DEX) is a long-acting potent synthetic GC with minimal mineralocorticoid activity that is administered orally, intravenously or by injection (Weijtens *et al.*, 1998; Wenting-Van Wijk *et al.*, 1999; Fardet and Fève, 2014). As with CORT, reported DEX serum concentrations (see **Table 1.3**) vary considerably. Prolonged exposure to GCs is associated with adverse local and systemic effects including central obesity, osteroporosis, hyperglycemia, diabetes, bone necrosis, delayed wound healing, and an increased risk to opportunistic infection as a result of prolonged immunosuppression (Liu *et al.*, 2013; Shäcke *et al.*, 2002). The majority of GR metabolic and adverse effects are mediated by transactivation (Shäcke *et al.*, 2002; Baschant and Tuckermann, 2010; Ramamoorthy and Cidlowski, 2013).

Table 1.3: **CORT and DEX peak serum concentrations**.

Glucocorticoid	Mode of administration	Serum concentration (nM)	Relative GR activity♦[6]	Relative MR activity♦[6]
CORT	Basal total	496 ± 132[1]	1	1
	Basal free	36.9 ± 17.2[1]		
	Major stress total	811.4 ± 268[1]		
	Major stress free	107.8 ± 56[1]		
Hydrocortisone	20 mg (oral)	179 ± 20•[2]		
	50 mg (intravenous)	694 ± 112•[2]		
DEX	3.75 mg (injection)	142.7 ± 48.4[3] (11.2 – 530.0)	30	0
	1 mg (oral)	11.7 ±5.9[4] (0.8 – 25.7)		
	7.5 mg (oral)	157+[5] (6.4 – 250.0)		

Serum concentrations are given as mean ± standard deviation unless otherwise indicated. Ranges are indicated in parentheses where available. References; [1] Christ-crain *et al.*, 2015; [2] Jung *et al.*, 2014; [3] Weijtens *et al.*, 1997; [4] Asvold *et al.*, 2012; [5] Weijtens *et al.*, 1998; [6] Nicolaides *et al.*, 2018. Symbols; [•] serum free concentration; [+] median; [♦] Relative binding affinities are relative to 100% for reference steroids for each steroid receptor; GR, hydrocortisone; MR, aldosterone.

1.6.9 *Mechanism of glucocorticoid-induced osteoporosis*

GCs regulate bone cell replication, differentiation and function (reviewed by Canalis and Delany, 2002). Long- term exposure to GCs, even at low physiological doses, is associated with increased risk of osteoporosis and bone fractures (Canalis and Delany, 2002; Lane, 2006). GC association with osteroporosis is thought to be through increased GC-induced expression of the cytokine receptor activator of NF-κB ligand (RANKL) and GC-induced repression of osteoprotegerin (OPG), which results in increased bone resorption (Canalis and Delany, 2002; Steeve *et al.*, 2004; Lane, 2006). The maintenance of healthy bone is dependent on both bone resorption by osteoclasts and bone formation by osteoblasts; when osteoclast activity exceeds that of osteoblasts, bone resorption exceeds bone formation, and osteoporosis develops (Canalis and Delany, 2002; Steeve *et al.*, 2004; Lane, 2006). Osteoclastogenesis is the complex multistage process which involves osteoclast commitment, differentiation and activation of immature osteoclasts (Canalis and Delany, 2002; Steeve *et al.*, 2004; Lane, 2006). RANKL expression by osteoblasts induces osteoclastogenesis, and inhibits osteoclast apoptosis through binding to its receptor RANK, expressed in cells of the monocyte lineages which differentiate into osteoclasts,

as well as in mature osteoclasts (Steeve *et al.*, 2004). OPG is a decoy receptor for RANKL, and through binding RANKL, is able to inhibit its actions via RANK and consequently, osteoclastogenesis (Steeve *et al.*, 2004).

1.7 *Rationale, aims and hypotheses of the current study*

HAART allows HIV-1 infection to be managed as a chronic disease, rather than a fatal infection (Deeks *et al.*, 2013), and HCs allow women control over their reproductive health, through timing of pregnancy and family planning (Sedgh *et al.* 2014). A large number of HIV-1 positive women therefore use HCs and HAART concurrently. The most commonly used progestin for HC in Sub-Saharan Africa is MPA (UN 2015; Hapgood *et al.*, 2018).

Biomedical HIV-1 preventative interventions targeted at women are under development, in an effort to reduce disease burden amongst women, who are disproportionately affected by the HIV-1 epidemic (Kelly and Shattock, 2011). These include MPTs, which are combination ARV-progestin combination products for the simultaneous prevention of HIV-1 infection and unintended pregnancy (Friend *et al.*, 2013; Doncel *et al.*, 2016). TDF is widely used in HAART, as well as oral PrEP, and is a leading candidate for use in microbicides and MPTs (Arts and Hazuda, 2012; Thurman *et al.*, 2013; Riddell *et al.*, 2018). DPV is an ARV developed for use in microbicides and MPTs, and so far it the only microbicide to demonstrate efficacy in two independent Phase III clinical trials (IPM, 2016). MVC is widely used in HAART, and was an early candidate for use in vaginal microbicides (Dorr *et al.*, 2005; Lieberman-Blum *et al.*, 2008; Chen *et al.*, 2015; Fletcher *et al.*, 2016). The progestin of choice in all MPTs under development is LNG (IPM, 2016).

Genital inflammation is a risk factor for HIV-1 acquisition in women and has been linked to the failure of early microbicides, some of which in fact increased HIV-1 acquisition in users (Fichorova *et al.*, 2004a, Naranbhai *et al.*, 2012, McKinnon, 2017). Moreover, chronic immune activation and inflammation are central to the pathogenesis of HIV-1 infection, drive HIV-1 disease progression, and may contribute to non-AIDS mortality (Ford, Puronen and Sereti, 2009; Cohen *et al.*, 2011). Therefore, to fully understand the effects of ARVs on HIV-1 disease progression when used therapeutically and on risk factors for HIV-1 acquisition when used prophylactically, it is important that their immunomodulatory effects, and the mechanisms thereof, be characterized. MVC TDF and DPV have been shown to have immunomodulatory effects *in vitro*, but the mechanisms through which they occur have not been determined (Gali *et*

al., 2010; das Neves *et al.*, 2013; Hladik *et al.*, 2015; Madrid-Elena *et al.*, 2018). It is conceivable some of the ARVs immunomodulatory effects are mediated via the GR; a ubiquitous ligand activated transcription factor which regulates all aspects of immune function (Baschant and Tuckermann, 2010; Yudt and Cidlowski, 2016).

It is clear the use of ARVs and progestins is widespread, and set to increase, but few studies have assessed reciprocal modulation of their intracellular activity (Nanda *et al.*, 2017). Those that have, have predominantly assessed ARVs effects on the pharmacodynamics, pharmacokinetics and contraceptive efficacy of progestins (Nanda *et al.*, 2017). The few studies that have assessed the effects of MPA on the anti-HIV-1 efficacy of TDF are contradictory (Heffron *et al.*, 2014; Shen *et al.*, 2017). Strikingly, Shen *et al.* showed MPA reduced the anti-HIV-1 activity of TDF in CD4+ T cells *in vitro* (Shen *et al.*, 2017). However, the mechanism through which this occurred was not determined. MPA use is associated with a significantly increased risk of HIV-1 acquisition, and its immunomodulatory effects via the GR, for which it is a partial agonist for transactivation, and a full agonist for transrepression, have been implicated as a contributing factor (Hapgood *et al.*, 2018). Shen *et al.* did not investigate whether MPA was acting via the GR in decreasing the anti-HIV-1 efficacy of TDF (Shen *et al.*, 2017). MPAs effects on the anti-HIV-1 efficacy of DPV have not been assessed. It is important the combined immunomodulatory effects of progestins and ARVs be investigated, to determine implications for HIV-1 susceptibility, off-target side effects and HIV-1 disease progression in individuals using ARVs and progestins concurrently.

Given the pleiotropic effects of GCs, ARVs are likely to be in the presence of these steroids nearly continuously intracellularly. However, whether GCs, produced endogenously, or used therapeutically, alter the antiviral efficacy of TDF, DPV, or other ARVs, has not been explored. Whether ARVs alter GC action via the GR has also not been evaluated. Intriguingly, some of the adverse effects observed in patients using TDF are similar to those in individuals on long-term GC therapy, for example, decreases in BMD and osteoporosis (Brown and Qaqish, 2006). Given that GCs acting via the GR regulate bone cell differentiation, replication and function, it is tempting to speculate these may occur in part through TDF transcriptionally activating the GR, or modulating GC activity via the GR. Given the critical role of GCs in regulating immunity, investigating the combined immunomodulatory effects of ARVs and GCs informs how ARVs may influence HIV-1 susceptibility and HIV-1 disease progression. It also informs on the off-target side effects which may be experienced in HIV-1 positive individuals using GCs therapeutically. Against this background, the current study evaluated the immunomodulatory

effects of DPV and TDF via the GR, a ubiquitous and critical regulator of immunity and inflammation, and virtually all other physiological processes (Baschant and Tuckermann, 2010).

The central hypothesis of this study is that TDF and DPV have off-target effects via the GR, either by activating the GR directly, or modulating the activity of GCs via the GR, which may explain their immunomodulatory effects, and some adverse effects reported with prolonged use of the ARVs. These effects may be mediated by altering GR activity, expression levels, and/or GR phosphorylation. The ARVs may also modulate the GR activity of the progestin MPA which is a full agonist/partial agonist for the receptor. GCs and MPA may modulate the antiviral efficacy of DPV and TDF, possibly through their immunomodulatory effects via the GR. Conversely, progestins with no GR activity, like LNG, would not modulate ARV anti-HIV-1 efficacy.

The aims of the current study are to:

i. **Determine if DPV and TDF have transcriptional activity via the GR**. This will be investigated in the U2OS human osteosarcoma cell line exogenously expressing the GR using GRE-luciferase reporter assays.
ii. **Determine if DPV and TDF can modulate the transcriptional activity of GCs and progestins via the GR.**. The ability of the ARVs, in the absence and presence of DEX, MPA and LNG, to modulate GRE-luciferase reporter gene transcription via the GR will be assessed in U2OS cells.
iii. **Determine if DPV and TDF have effects on GC- and progestin-induced endogenous GR-regulated mRNA expression**. The effects of the ARVs on DEX-, CORT-, LNG- and MPA-induced gene expression will be investigated in U2OS cells. The mRNA expression of the select GR transactivated genes GILZ and IκB-α will be assessed.
iv. **Determine the effects of DPV and TDF on GR protein levels and GR phosphorylation**. The effects of the ARVs, in the presence and absence of DEX will be investigated in the U2OS cell line model. Total GR protein levels, and phosphorylation at Ser226 on the GR will be assessed by western blotting.
v. **Determine the effects of DPV and TDF on immunomodulatory gene expression, alone and in the presence of GC in peripheral blood mononuclear cells.** The effects of the ARVs on DEX-induced mRNA expression will be investigated in PBMCs from

female donors. The mRNA expression of select GR-regulated anti-inflammatory genes GILZ and IκB-α, and proinflammatory genes IL-6, IL-8 and IFN-γ will be assessed.

vi. **Determine the effects of GC and progestins on the HIV-1 inhibitory effects of DPV and TDF.** The effects of DEX, MPA and LNG on the ARVs anti-HIV-1 efficacy will be investigated in PBMCs.

Secondarily, the ability of MVC to activate the GR, and modulate the transcriptional activity of DEX, MPA and LNG will be assessed in COS-1 cells using luciferase promoter-reporter assays. The immunomodulatory effects of MVC in PBMCs, in the absence and presence of DEX will be compared to those of DPV and TDF.

U2OS human osteosarcoma cells are steroid-deficient cells (Niforou *et al.*, 2008), with a high transfection efficiency (Tang *et al.*, 2005). As such, they are well suited to exploring the first five aims of this study, outlined above. The low level of steroid receptors in U2OS cells allows the effects of ARVs in the presence and near-absence of GR to be assessed. In addition, interpretation of any effects observed is not confounded by the presence of other steroid receptors. GILZ is strongly induced by GCs, and a critical mediator of the anti-inflammatory and immunosuppressive effects of GCs (Riccardi, 2010). Through inhibiting the action of AP-1 and NF-κB, it prevents the transcription of pro-inflammatory cytokines like IL-6 and IL-8, and in macrophages, GILZ prevents activation (Berrebi *et al.*, 2003; Riccardi, 2010). IκB-α, like GILZ, is regulated by GR transactivation, and has been shown to be rapidly upregulated by GCs (Deroo and Archer, 2001). IκB-α has been shown to cause cytoplasmic retention of NF-κB by sequestering nuclear localization signals essential for nuclear import (Jacobs and Harrison, 1998), inducing export of NF-κB from the nucleus, inhibiting DNA binding by NF-κB, and inhibition of cAMP-dependent protein kinase, whose actions are necessary for NF-κB nuclear translocation (Ghosh *et al.*, 1998).

IL-6 and IL-8 are proinflammatory cytokines, regulated by GR transrepression (Baschant and Tuckermann, 2010; Ramamoorthy and Cidlowski, 2013). IL-8 is a potent chemoattractant of neutrophils to sites of inflammation (Bickel, 1993). Elevated IL-8 levels have been reported in the lymphoid tissue of HIV-1 positive, and AIDS patients (Lane *et al.*, 2001). The role of IL-8 in HIV-1 replication and disease progression is somewhat controversial, with several contradictory reports. For example, Capobianchi *et al.* reported no effect of IL-8 on HIV-1 replication in monocyte-derived macrophages (Capobianchi *et al.*, 1998), while another study reported a slight inhibitory effect of IL-8 on HIV-1 replication in CD4+ T cells (Mackewicz *et al.*, 1994). In contrast,

IL-8 has also been reported to increase gene expression from the HIV-1 long terminal repeat (Marechal *et al.*, 1999), and to dose-dependently stimulate HIV-1 replication in macrophages and T cells *in vitro* (Lane *et al.*, 2001). IL-6 is rapidly produced at sites of infection, and, as well as inducing differentiation of activated B cells into antibody producing cells, induces production of acute phase proteins in hepatocytes (reviewed by Tanaka *et al.*, 2014). Acute phase proteins amplify local inflammation by enhancing the detection of pathogens, promoting leukocyte recruitment into blood circulation and increasing blood flow to sites of infection (Jain *et al.*, 2011). However, IL-6 also has anti-inflammatory properties; endogenous IL-6 may dampen local and acute inflammatory responses by modulating proinflammatory (but not anti-inflammatory) cytokine levels (Xing *et al.*, 1998). Elevated levels of pro-inflammatory cytokines, including IL-6 and IL-8 have recently been shown to have undermined the effectiveness of a TFV microbicidal gel in preventing HIV-1 acquisition in women (Mckinnon *et al.*, 2018). Furthermore, early failed microbicide candidates which increased HIV-1 infection also elevated IL-8 levels (Fichorova *et al.*, 2004a, 2004b; Naranbhai *et al.*, 2012, McKinnon, 2017). IFN-γ has broad and varied immunoregulatory effects, including inducing lymphocyte activation, antigen presentation and pro-inflammatory antiviral responses (Roff *et al.* 2013). Although its ability to inhibit HIV-1 replication in monocytes and macrophages (Kornbluth *et al.*, 1990; Roff *et al.* 2013) may be protective against HIV-1 infection *in vivo,* it's pro-inflammatory effects and effects on lymphocyte activation may be detrimental (Roff *et al.* 2013). As immune responses are influenced by sex, only PBMCs from female donors were used in this study (Klein and Flanagan, 2016).

CHAPTER 2

MATERIALS AND METHODS

2.1 *Ethics and biosafety*

This research formed part of a sub-study of HREC 210/2011, which was approved by the Human Research Ethics Committee at the University of Cape Town. Approval included the use of human blood from the Western Province Blood Transfusion Services. All procedures were sanctioned and carried out in accordance with established guidelines. This study adhered to the biosafety procedures established by the Health and Safety Committee of the Department of Molecular and Cell Biology at the University of Cape Town.

2.2 *Test compounds*

The ARVs 4-[[4-(2,4,6-trimethylanilino)pyrimidin-2-yl]amino]benzonitrile (dapivirine, DPV) and 9-[(R)-2-[[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy]propyl]adenine fumarate (tenofovir disoproxil fumarate, TDF) were purchased from Selleck Chemicals (USA). 4,4-Difluoro-N-[(1R)-3-[(1R,5S)-3-(3-methyl-5-propan-2-yl-1,2,4-triazol-4-yl)-azabicyclo[3.2.1]octan-8-yl]-1-phenylpropyl]cyclohexane-1-carboxamide (maraviroc, MVC) was obtained from the NIH AIDS Reagent Program (USA). DPV, TDF and MVC were dissolved in dimethyl sulfoxide (DMSO) from Sigma-Aldrich (South Africa), to a stock concentration of 10 mM, which was further serially diluted. The steroids (11b,16a)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione (dexamethasone, DEX), (11beta)-11,17,21-trihydroxypregn-4-ene-3,20-dione hydrocortisone (cortisol, CORT), 6α-methyl-17α-hydroxy-progesterone acetate (medroxyprogesterone acetate, MPA) and 13β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one (levonorgestrel, LNG) were obtained from Sigma-Aldrich (South Africa), and were prepared in absolute ethanol (EtOH). ARVs and steroids were added to cells such that all incubations contained 0.1% (*v*/*v*) DMSO or EtOH respectively. Where cells were treated with combinations of steroids and ARVs, all incubations contained 0.1% (*v*/*v*) DMSO and 0.1% (*v*/*v*) EtOH. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was procured from Sigma Aldrich (South Africa).

2.3 *Cell culture*

U2OS human osteosarcoma cells and COS-1 African green monkey kidney fibroblast-like cells were purchased from America Type Culture Collection (USA). Cells were cultured in 75 cm^2 flasks (Greiner Bio-One International, Austria) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 1 mM sodium pyruvate (Sigma-Aldrich, South Africa), 44 mM sodium bicarbonate (Sigma-Aldrich, South Africa), 10% (*v*/*v*) foetal bovine serum (FBS) (Thermo Scientific, South Africa), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, Invitrogen, UK), from hereon referred to as full DMEM. In all experiments, cells were cultured in a water jacketed incubator at 37°C, 90% humidity and 5% CO_2. To sub-culture, cells were washed with warm 1 × phosphate-buffered saline (PBS, Sigma Aldrich, South Africa) and incubated at 37°C with 2 mL 0.25% trypsin/0.1% EDTA in PBS (Highveld Biological, South Africa) for 3 min. Trypsin was neutralized by the addition of full DMEM. Cells were routinely checked for mycoplasma contamination by Hoescht staining and fluorescence microscopy (Freshney, 2010), with only mycoplasma-negative cells utilized in experiments. Average and maximum passage numbers were approximately 30 and 40, respectively, for COS-1 cells, and 50 and 65, respectively, for U2OS cells. For all experiments performed in this study using cell lines, independent biological repeats were performed on different days, using cells which had been passaged at least once since their previous use.

2.4 *Plasmids, plasmid transformation and purification*

The empty vector, pcDNA3.1, containing a cytomegalovirus promoter and lacking further downstream DNA sequence, was obtained from Invitrogen (UK). The glucocorticoid expression vector pcDNA3-hGR, comprising full-length human GRα cloned into the pcDNA3 vector, was a gift from D. W. Ray (University of Manchester, UK) (Ray *et al.*, 1999). The luciferase reporter plasmid, pTAT-GRE-E1b-LUC, containing two GREs from the rat tyrosine amino transferase gene (TAT), regulated by the E1b promoter was a gift from G. Jenster (Erasmus University of Rotterdam, Netherlands) (Sui *et al.*, 1999).

Plasmids were transformed into *Escherichia coli* DH5α cells using heat shock as previously described (Sambrook and Russell, 2006). Briefly, competent DH5α cells (100 µL) were incubated on ice for 30 min with 10 ng plasmid DNA. Thereafter, cells were incubated at 42°C for 2 min and immediately placed on ice for 2 min. Following this, 900 µL Luria broth (LB, 1% (*w*/*v*) tryptone,

0.5% (*w*/*v*) yeast extract, 0.5% (*w*/*v*) NaCl) was added, and the mixture incubated on an orbital shaker at 37°C for 1 hour. Transformed cells were then plated onto LB-agar plates (1% (*w*/*v*) tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) containing 100 µg/mL ampicillin (Sigma Aldrich, South Africa) and incubated overnight at 37°C. To confirm selection of transformed cells, cultures were also plated onto LB-agar plates not containing ampicillin. Day cultures of 5 mL LB containing 100 µg/mL ampicillin were inoculated with single colonies and incubated at 37°C for 8 hours with shaking. Overnight cultures of 200 mL LB containing 100 µg/mL ampicillin inoculated with 200 µL day culture cell suspension were incubated at 37°C on an orbital shaker for 16 hours. The NucleoBond® Xtra Maxi plasmid DNA purification kit (Macherey-Nagel) was used for plasmid purification according to manufacturer's instructions. The purity and yield of the extracted plasmids were evaluated using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). Samples were electrophoresed on a 1 × Tris-Acetate-EDTA (TAE) 1% agarose gel containing 10 µg/mL ethidium bromide (Sigma Aldrich, South Africa) at 100 V for 1 hour to assess plasmid integrity, conformation, and RNA contamination. Samples were visualized under ultraviolet light using a Syngene G:Box (Vacutec, UK) and images acquired using GeneSnap version 7.08 (SynGene, UK).

2.5 *Restriction enzyme digests*

Plasmid identity and integrity was confirmed by restriction enzyme digest. Identity was confirmed by assessing restriction enzyme banding patterns. Each restriction enzyme digest mixture comprised 300 ng of DNA, 1 U/mL of the restriction enzyme (or an equivalent volume of nuclease free water (Thermo Scientific, USA) for undigested controls), 1 × Fast Digest buffer (Fermentas, Thermo Scientific, USA) and nuclease free water to a final volume of 20 µL. The reaction mixture was incubated for 10 min at 37°C. The resulting digests were electrophoresed on a 1 × TAE 0.8% agarose gel containing 10 µg/mL ethidium bromide (Sigma Aldrich, South Africa) at 100 V for 1 hour. Samples were visualized under ultraviolet light using a Syngene G:Box (Vacutec, UK) and images acquired using GeneSnap version 7.08 (SynGene, UK).

2.6 *Plasmid transfection*

For promoter-reporter assays, 1.5×10^6 U2OS cells were seeded into 10 cm dishes (Greiner Bio-One International, Austria) in full DMEM. After a 24-hour incubation, to ensure consistent transfection efficiency cells were transiently bulk-transfected with 10 µg pcDNA3-hGR or the

empty vector pcDNA3.1 as a negative control and 3.75 μg pTAT-GRE-E1b-LUC using X-tremeGENE 9 (Roche, South Africa) according to the manufacturer's instructions. The use of lower concentrations (2.5 μg and 5 μg) of GR expression vector was initially tested, but GR-mediated responses in these experiments were not robust (data not shown). Where endogenous GR-regulated mRNA expression was determined, cells were transfected as above, with the exclusion of pTAT-GRE-E1b-LUC.

2.7 *Luciferase reporter assays*

U2OS cells were transiently bulk-transfected with the GR expression vector pcDNA3-hGR and luciferase reporter pTAT-GRE-E1b-LUC as specified above. After 24 hours, cells were trypsinized and seeded into 96-well plates (Greiner Bio-One International, Austria) at a density of 1×10^4 cells per well in 200 μL full DMEM. Following a 24-hour incubation, cells were washed with warm 1 × PBS. To evaluate the activity of DPV and TDF via the GR, cells were stimulated with DPV and TDF at concentrations ranging from 0.01 nM – 10 μM, in the absence or presence of 100 nM DEX in serum free DMEM (DMEM supplemented as indicated elsewhere in this chapter, with the exclusion of FBS) for 24 hours. To evaluate the capacity of DPV and TDF to modulate steroid activity via the GR, cells were treated with DEX, MPA or LNG at the concentrations indicated in figures, in the presence or absence of 1 μM DPV or TDF for 24 hours. Thereafter, cells were washed with ice cold 1 × PBS and harvested in 25μL 1 × luciferase reporter lysis buffer by shaking on a rotating shaker following a freeze-thaw step at -21°C. Luciferase activity was assessed using the Luciferase Assay System (Promega, USA). Relative light units (RLU) in 10 μL cell lysate upon the addition of 50 μL luciferin substrate in white 96-well plates (Greiner Bio-One International, Austria) were determined using a Modulus microplate luminometer (Turner Biosystems, USA). Luciferase activity was normalized to protein content per well, as determined by a Bradford assay (Bradford, 1976). To evaluate the activity of MVC via the GR, the same procedures outlined above were performed using COS-1 African green monkey kidney fibroblast-like cells, purchased from America Type Culture Collection (USA). The number of experimental repeats is indicated in figure legends throughout this text. `

2.8 *PBMC isolation and culture*

Whole blood from healthy female donors was obtained from the Western Province Blood Transfusion Services (Cape Town, South Africa). Donors tested negative for HIV-1, syphilis and

hepatitis B and C. PBMCs were isolated by Histopaque (H1077 Hybri-Max, Sigma-Aldrich, South Africa) density centrifugation using Leucosep tubes (Greiner Bio-One International, Austria). At room temperature, 15 mL Histopaque was centrifuged in Leucosep tubes at 400 × g for 1 min using a Heraeus Megafuge 40 centrifuge (Thermo Scientific, South Africa). Whole blood was diluted in unsupplemented Roswell Park Memorial Institute (RPMI) 1640 (Lonza, Switzerland) and PBS at a ratio of 2:1:1 in 50 mL tubes, following which it was transferred to the Leucosep tubes and centrifuged at 400 × g for 15 min without brakes. The white buffy coat containing separated PBMCs was collected and washed twice with 1 × PBS supplemented with 1% (*v*/*v*) charcoal stripped (cs)-FBS by centrifugation at 250 × g for 5 min. Cell number and viability was determined by trypan blue staining, where PBMCs were added to trypan blue solution (Lonza, Switzerland) at a ratio of 1:10. PBMCs were then added to a freezing media comprised of 20% DMSO (*v*/*v*) in cs-FBS and stored at -80°C until required.

PBMCs were cultured in RPMI 1640 media (Lonza, Switzerland), supplemented with 10% (*v*/*v*) cs-FBS (Thermo Scientific, South Africa), 2mM L-glutamine (Sigma-Aldrich, South Africa), 100 U/mL penicillin and 100 mg/mL streptomycin. (Gibco Invitrogen, UK) and 30 U/mL IL-2 (Gentaur, Belgium) (from here on termed full RPMI), at 37°C. For experiments, frozen PBMC stocks were thawed and seeded into sterile 50 mL V-bottomed tubes (Greiner Bio-One International, Austria), at a concentration of 1 million cells/mL in 45 mL full RPMI. Cells were incubated overnight, following which PBMCs were pelleted and washed twice by centrifugation at 250 × g in 1 × PBS supplemented with 1% (*v*/*v*) cs-FBS. Cells were seeded into 5 mL Falcon tubes (Becton Dickson Scientific, South Africa) at a density of 2 million cells in 2 mL full RPMI. Subsequently, PBMCs were stimulated with ARVs and steroids for 48 hours as indicated in figure legends. Thereafter, PBMCs were pelleted by centrifugation at 250 × g for 5 min and harvested in 400 µL TriReagent® (Sigma-Aldrich, South Africa) for RNA isolation. To ensure oxygenation of PBMC media, the lids of falcon tubes were left partially unscrewed.

2.9 *Viral propagation*

HEK293T human embryonic kidney cells (America Type Culture Collection, USA) were seeded into 10 cm plates at a density of 4×10^6 in full DMEM and incubated overnight at 37°C, 90% humidity and 5% CO_2. Subsequently media was replaced and cells were transfected with 12 µg HIV-$1_{BaL\text{-}Renilla}$ or a control (DMEM) using X-tremeGENE 9 DNA transfection reagent in

accordance with the manufacturer's instructions. HIV-$1_{BaL\text{-}Renilla}$ is an infectious, replication-competent molecular clone created by insertion of a luciferase gene next to *env* in the HIV-1 NL4-3 backbone, NL-LucR.T2A-BaL.ecto, and was a gift from Dr. Christina Ochsenbauer (Edmonds *et al.*, 2010). Following a 48-hour incubation, the media was harvested and passed through a 0.22 μm filter. cs-FBS was added to the filtered media to a final concentration of 12.5%. This was then aliquoted into 2 mL cryovial tubes (Nunc, Germany), and stored at -80°C until required. Viral titres were determined using the TZM-bl assay as described in (Edmonds *et al.*, 2010). After a 72-hour incubation, cells were harvested in 120 μL Bright-Glo luciferase lysis buffer (Promega, USA) and transferred to white 96-well plates (Greiner Bio-One International, Austria) and luminescence was determined on a luminometer (Modulus Microplate, Promega, USA). The titre of the virus stock was determined using the Reed-Muench method and expressed as log infectious units (IU)/mL (Reed and Muench, 1938).

2.10 *PBMC infection assays*

PBMCs were prescreened for infectability, owing to the high inter-donor variability in HIV-1 infection observed in the laboratory of the present author, following which they were stored at -80 degrees. Thereafter, infectible PBMCs were thawed and seeded into sterile 50 mL V-bottomed tubes (Greiner Bio-One International, Austria), at a concentration of 1 million cells/mL in 45 mL full RPMI. Cells were incubated overnight and subsequently, 2 million cells were seeded into 5 mL Falcon tubes (Becton Dickson Scientific, South Africa) and stimulated with ARVs and steroids for 48 hours as indicated in figure legends. Following this, PBMCs were infected with 10 IU/mL HIV-$1_{BaL\text{-}Renilla}$ or an equivalent volume of no virus control, in the presence of the ARVs and steroids (multiplicity of infection= 5×10^{-6}) and incubated for 2 hours. No dose-response experiments for infection with HIV-$1_{BaL\text{-}Renilla}$ were performed. PBMCs were then pelleted by centrifugation at 800 × g for 5 min and washed 3 times in 1 × PBS supplemented with 1% cs-FBS. After the addition of 2 mL full RPMI, cells were incubated for 5 days, with no additional change in media. Following this, PBMCs were transferred into two 96-well U-bottom plates (Greiner Bio-One International, Austria) in quadruplicate, at a density of 2×10^5 cells per well. Into one plate, 20 uL filter sterilized MTT solution (5 mg/mL MTT in PBS) was added, to a final concentration of 0.5 mg/mL and incubated for 2 hours. Cells were pelleted by centrifugation at 250 × g for 5 min and resuspended in 70 μL solubilizing solution (0.1 N HCl in isopropanol) per well, 50 μL of which was transferred into a new 96-well plate. Cell viability was determined by measuring absorbance at 595 nm on a spectrophotometer (Thermo Scientific, USA). Cells in the second plate

were pelleted by centrifugation at 250 × g, and were harvested to determine infection, represented by *Renilla* luciferase expression using *Renilla* luciferin according to the manufacturer's specifications (Promega, USA). Briefly, PBMCs were resuspended in 70 μL/well 1 × reporter lysis buffer with *Renilla* luciferin after incubation for 10 min in the dark at room temperature. Lysed cells were transferred into a white 96-well plate (Greiner Bio-One International, Austria) and luminescence in RLU was measured on a luminometer (Modulus Microplate, Promega, USA). Infection was calculated by dividing the RLU obtained for each sample by the average MTT absorbance value for that sample group. Relative infection was then calculated by setting vehicle control (EtOH + 0.1% (*v*/*v*) DMSO) to 100% infection.

2.11 *Western blotting and antibodies*

For positive controls, COS-1 cells were seeded into 12-well plates (Greiner Bio-One International, Austria) at a density of 1 × 10^5 cells per well and incubated for 24 hours. Thereafter, cells were transiently transfected with 1 μg/well of the GR expression vectors using X-tremeGENE 9 and incubated for 24 hours. Cells were then washed in ice-cold 1× PBS, lysed with 50 μL 2 × SDS sample buffer (5 × SDS sample buffer: 100 mM Tris pH 6.8, 5% *v*/*v* SDS, 20% *v*/*v* glycerol, 5% *v*/*v* β-mercaptoethanol, 0.1% *w*/*v* bromophenol blue), then boiled at 100°C for 10 min and stored at -20°C until use. For experiments, following transfection of U2OS cells with the GR expression vector as described in Section 2.6, cells were trypsinized and seeded into 12-well plates (Greiner Bio-One International, Austria) at a density of 1 × 10^5 cells per well. Cells were stimulated as indicated in figure legends for 24 hours, after which they were washed in ice cold 1 × PBS, lysed with 50 μL 2 × SDS sample, boiled at 100°C for 10 min and stored at -20°C until use. Equal volumes of lysate were loaded on a 10% SDS-polyacrylamide gel. Samples were separated by electrophoresis at 75 V for 25 min then 150 V for 1 hour in 1 × running buffer (25 mM TRIS-HCl, 250 mM glycine and 0.1% (*v*/*v*) SDS pH 8.4) using a Bio-Rad Mini Protean II electrophoresis system (Bio-Rad, South Africa). Subsequently, sample was transferred onto a Hybond-ECL nitrocellulose membrane (Amersham, South Africa) for 1 hour at 0.18 A in ice cold 1 × transfer buffer (25 mM TRIS, 200 mM glycine, 20% (*v*/*v*) methanol). Subsequently, nitrocellulose membranes were blocked in 4% (*w*/*v*) ECL blocking reagent powder in 1 × TRIS-buffered saline (50 mM TRIS, 150 mM NaCl, pH 7.6; TBS) containing 0.1% (*v*/*v*) Tween (TBS-Tween; TBST) for 1 hour. Nitrocellulose membranes were then incubated in 10 mL of 4% ECL-TBST containing primary antibodies and shaken gently overnight at 4°C. Primary and secondary antibodies are

detailed in **Table 2.1** below. Following this, membranes were washed 2 times in 1 × TBST for 5 min and once for 10 minutes. Membranes were then incubated at room temperature with secondary antibodies diluted in 5% (*w*/*v*) skim milk powder in 1 × TBST, on a rotating shaker for 1 hour. After three washes in 1 × TBST as above, membranes were placed in 1 × TBS followed by a 1 min incubation with Pierce ECL-chemiluminescent western blotting substrate (Thermo Scientific, USA). Proteins were visualized by autoradiography using Amersham Hyperfilm™ MP high performance autoradiography film (AEC-Amersham, South Africa). Densitometric quantification of film was carried out using ImageJ software (NIH, USA).

Table 2.1: **Primary and secondary antibodies used for Western blotting**

Primary Antibody	**Detected Size (kDa)**	**Primary Antibody Dilution**	**Secondary Antibody**	**Secondary Antibody Dilution**
GRα (G-5, sc-393232; Santa Cruz Biotechnology)	95	1:4000	Anti-mouse HRP linked antibody (sc-516102; Santa Cruz Biotechnology)	1:4000
Phosho Ser226-GR (D9D3V, #97285 Cell Signaling Technology)	95	1:3000	Anti-rabbit HRP linked antibody (#7074; Cell Signaling Technology)	1:3000
GAPDH (0411, sc-47724, Santa Cruz Biotechnology)	37	1:2000	Anti-mouse HRP linked antibody (sc-516102; Santa Cruz Biotechnology	1:1000

2.12 *RNA isolation and cDNA synthesis*

After the addition of 400 µL TriReagent® (Sigma-Aldrich, South Africa), 12-well plates (Greiner Bio-One International, Austria) were scraped and the contents transferred into microfuge tubes. Following the addition of 80 µL chloroform, tubes were vortexed vigorously for 30 seconds and incubated at room temperature for 3 min, after which they were centrifuged at 20 000 × g at 4°C for 15 min. The aqueous phase was then transferred into a new microfuge tube, into which 200 µL isopropanol (Merck, South Africa) and 1 µL glycogen (Roche, South Africa) was added. After mixing by inversion, samples were incubated for 15 min at room temperature. Samples were then centrifuged at 20 000 × g at 4°C for 10 minutes. The supernatants were discarded and the pelleted RNA washed in 400 µL of 75 % (*v*/*v*) EtOH in diethyl pyrocarbonate (DEPC)-treated water.

Subsequently, the RNA pellet was air dried and resuspended in 20 µL DEPC-treated water. Samples were then incubated at 55°C for 5 min and immediately placed on ice. RNA was precipitated by the addition of 40 µL 100% (*v*/*v*) EtOH and 6 µL 3 M sodium acetate pH 5.5 and stored at -80°C overnight. Following this, RNA was pelleted by centrifugation at 20 000 × g for 15 min at 4°C, washed in 700 µL of 75 % (*v*/*v*) EtOH in DEPC-treated water, air dried and resuspended in DEPC-treated water as above. RNA concentration and integrity was then determined by spectrophotometry (NanoDrop Technologies). Minimum acceptable 260:280 and 260:230 ratios were both approximately 1.8. Integrity was further assessed by denaturing formaldehyde agarose gel electrophoresis (Sambrook and Russell 2006). Briefly, 250 ng RNA was added to sample loading buffer [12% (*v*/*v*) DEPC-treated water, 5% (*v*/*v*) bromophenol blue, 7% (*v*/*v*) glycerol, 10% (*v*/*v*) 10 × MOPS buffer (0.2 M MOPS in DEPC-treated water, 0.05 M sodium acetate, 0.01 M EDTA), 17% (*v*/*v*) 12.3 M formaldehyde and 49% (*v*/*v*) formamide] and 20 µg/mL EtBr. Following this, samples were electrophoresed on a 1% formaldehyde agarose gel (70% (*v*/*v*) DEPC-treated water, 10% (*v*/*v*) 10 × MOPS buffer, 20% (*v*/*v*) formaldehyde) in 1 × MOPS buffer (40 mM MOPS; 10 mM sodium acetate; 1 mM EDTA, pH 8.00) at 70 V for 35 min. Samples were visualized under ultraviolet light on a Syngene, G:Box (Vacutec, UK) and images acquired using GeneSnap version 7.08 (SynGene, UK). 250 ng RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Science, South Africa). RNA samples were precipitated and stored at -80°C.

2.13 *Primers and Real-Time PCR*

For quantitative real-time polymerase chain reaction (qRT-PCR), equal volumes of synthesised cDNA were used as a template using the FastStart Essential DNA Green Master kit (Roche Applied Science, South Africa) and the primers listed in **Table 2.2**. Analysis was performed on a RotorGene 3000 qRT-PCR machine (Qiagen, Netherlands). Cycling parameters consisted of an initial 10 min denaturation at 95°C, 35 cycles of 10 seconds at 95°C, 15 seconds annealing at 60°C and 10 seconds elongation at 72°C. For IκB-α, cycling parameters were as above, with the inclusion of a final 10 min extension at 72°C. mRNA transcript levels were normalized to GAPDH mRNA transcript levels, and calculated using the Pfaffl method (Pfaffl, 2001). It was determined that the standard deviation of Ct values for GAPDH between different treatment and replicate samples was less than 1, indicating its suitability for use as a reference gene in both PBMCs and U2OS cells. RT-PCR product samples were analysed by 2% agarose gel electrophoresis in 1 × TAE at 70 V for 1 hour, to check for correct expected product sizes. The primer efficiency was

determined by standard curves. Fold change was calculated relative to the vehicle control set to 1, or as indicated in figures.

Table 2.2 : **Sequences, amplicon sizes and concentrations of primers used for real time-PCR**

Gene	Sequence	Product Size (bp)	Final Conc.	Reference
GAPDH	F: 5' -TGAACGGGAAGCTCACTGG- 3' R: 5' -TGTCAGTTGATAAAACCGCTGCC- 3'	307	200 nM	(Ishibashi *et al.*, 2003)
GILZ	Quantitect Primer QT00091035	69	1X	Qiagen, Netherlands
IL-6	F: 5' -TCTCCACAAGCGCCTTCG- 3' R: 5' -CTCAGGGCTGAGATGCCG- 3'	193	250 nM	(Wolf *et al.*, 2002)
IL-8	F: 5' -TGCCAAGGAGTGCTAAAG- 3' R: 5' -CTCCACAACCCTCTGCAC- 3'	197	500 nM	Wolf *et al.*, 2002)
IFN-γ	F: 5' -AATGCAGGTCATTCAGATGTAGCGG- 3 ' R: 3' -GGATGAGTTCATGTATTGCTTTGCG- 3'	298	250 nM	(Scott *et al.*, 1999)
IκB-α	F: 5' -ACTCGTTCCTGCACTTGGCC- 3' R: 5' -TGCTCACAGGCAAGGTGTAG- 3'	238	200 nM	(Emmerich *et al.*, 1999)

2.14 *Data analysis*

Data was analysed using GraphPad Prism (version 7) software from GraphPad Software Inc. (La Jolla, California, USA). For dose-response curves, DEX were used as the reference ligand and set to 100% by subtracting the average control reading from all other conditions, unless otherwise specified in figure legends. Dose-response curves were plotted using a non-linear regression model using "log agonist vs response", with a fixed Hill slope of 1. Statistical analysis is described in figure legends, and data was plotted as mean ± standard error of the mean (SEM).

CHAPTER 3
RESULTS

The ability of DPV and TDF to independently transcriptionally activate the GR, was assessed by reporter gene analysis in U2OS cells exogenously expressing the GR. In addition, their capacity to modulate GC-induced mRNA levels via the GR was similarly investigated in these cells. Their effects on GR-regulated mRNA levels of immunoregulatory genes was investigated in PBMCs, as were the effects of DEX, MPA and LNG on the efficacies of DPV and TDF in inhibiting HIV-1 replication. Concentrations of ARVs within the range of those detected intravaginally, as well as peak serum concentrations following oral administration, were used (see **Table 1.2**). Concentrations of GCs and progestins were also within physiologically relevant ranges (see **Table 1.1** and **Table 1.3**).

3.1 *DPV and TDF do not independently transcriptionally activate the GR*

To evaluate the capacity of DPV and TDF to activate the GR, U2OS cells exogenously expressing the GR (+GR) and a GRE-luciferase reporter were stimulated with DPV (**Fig. 3.1c**) or TDF (**Fig. 3.1e**), at concentrations ranging from 0.01 nM–10 μM, in the absence and presence of 100 nM DEX. In the absence of DEX, neither DPV nor TDF significantly induced the reporter above basal levels at the concentrations tested, suggesting they do not independently activate the GR. At a concentration of 10 μM, TDF (p>0.999) to induced a non-statistically significant increase in reporter gene expression of approximately 25-fold relative to vehicle. This represents the average of three values, one of which was much higher than the others, possibly due to a technical error. The addition of 100 nM DEX significantly induced the reporter by approximately 200-fold relative to vehicle, with no significant differences observed in the presence of DPV or TDF at any of the concentrations tested. In cells transfected with the empty vector control (-GR) (**Fig. 3.1b** & **d**), as expected, neither DEX nor the ARVs significantly induced the luciferase reporter above basal levels. Successful GR transfection was confirmed by western blotting (**Fig. 3.1a**). GR protein was not detected in cells transfected with the empty vector control, consistent with the lack of DEX-induced luciferase activity observed. Protein levels of other steroid receptors beyond the GR were not assessed directly by the author; however, others in the authors laboratory have shown (by western blotting) that U2OS cells do not express detectable levels of AR, PR or MR protein (data not shown). Upon agonist binding, it has been shown that GR levels in cells rapidly decline

(Wallace and Cidlowski, 2001; Avenant *et al.*, 2010b). Predictably DEX, unlike its vehicle control, significantly reduced GR protein levels in cells exogenously expressing the GR, by approximately 40% (**Fig. 3.1a**). Recognizing that the relatively high concentration of DEX used may overwhelm any modulatory effect of the ARVs in U2OS cells, similar experiments, using 10-fold lower concentrations of DEX were performed (data not shown). However, in these experiments, DEX-induced effects via the GR were not robust or reproducible. Unlike DEX, LNG (100 nM) did not significantly induce the reporter above basal levels, which remained unchanged in the presence of DPV or TDF (at concentrations of 100 nM and 1 μM) (Appendix **Fig. A1a & c**). DPV and TDF also had no significant effect on the transcriptional activity of MPA, which induced a non-statistically significant increase in reporter gene expression of approximately 100-fold relative to vehicle (Appendix **Fig. A1b & d**).

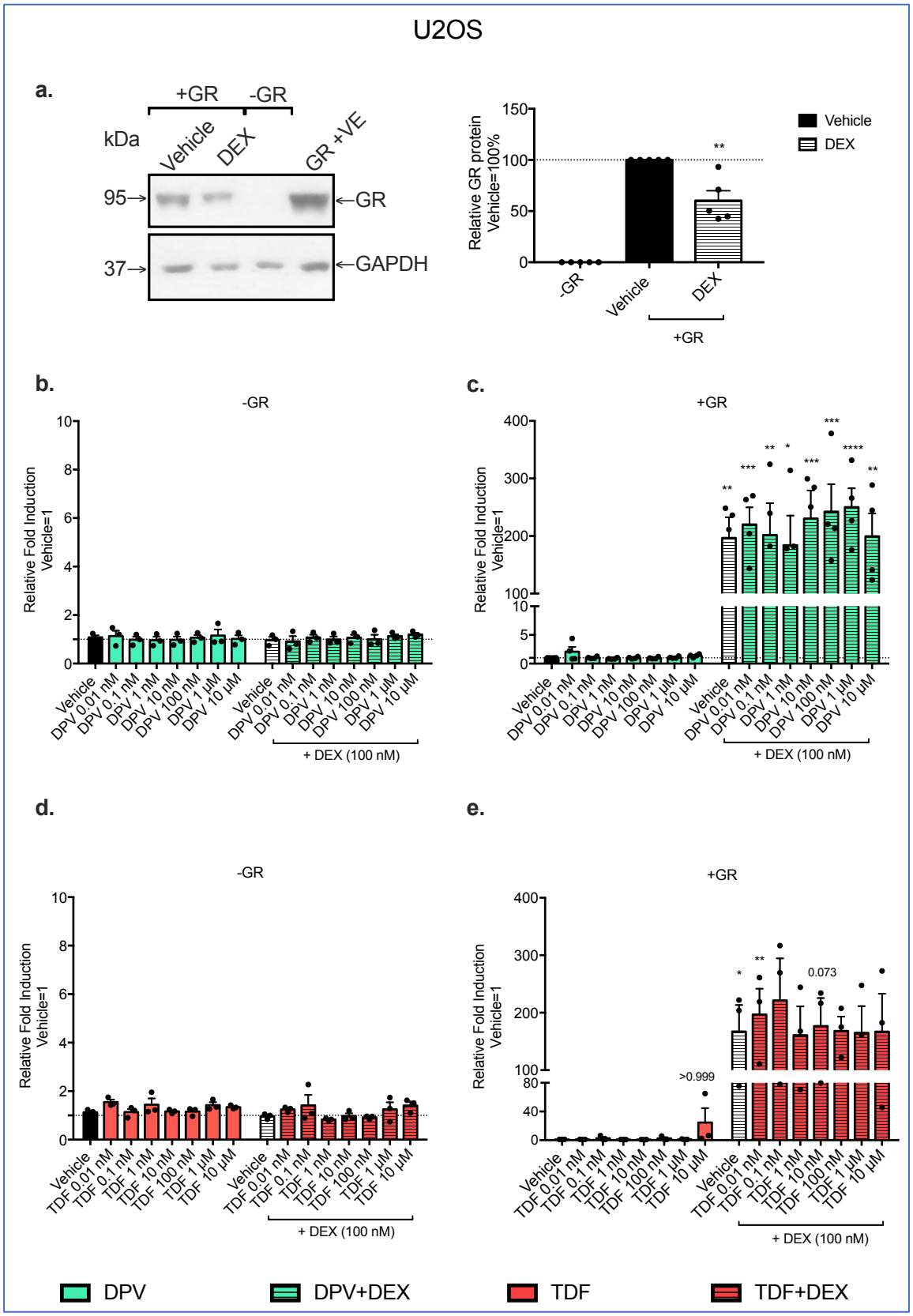

Figure 3.1: **DPV and TDF do not independently activate the GR.** U2OS cells were co-transfected with the GR expression vector (+GR) or its empty vector control (-GR), and the GRE-luciferase reporter construct. Equal volumes of cell lysate, including a GR positive control (GR +ve) were analysed by western blotting with a GR-specific antibody, and GAPDH as a loading control, to confirm successful GR transfection. A representative western blot is shown in the left panel and pooled results of five experiments

in the right panel of (**a**). Subsequently, cells were treated with increasing concentrations of DPV (**b**) and (**c**) or TDF (**d**) and (**e**), in the absence or presence of 100 nM DEX, or vehicle (0.1% *v*/*v* EtOH and DMSO) for 24 hours. Luciferase activity was normalized to protein content per well, as determined by a Bradford assay. Data are plotted as mean ± SEM, and are the pooled results of three or more independent experiments, each performed in triplicate. Data is plotted relative to fold induction for vehicle control (in the absence of DEX, black bar), which was set to 100% in (**a**) and 1 in (**b–e**). Statistical significance was determined using unpaired t-tests in (**a**) and a two-way ANOVA with a Tukey's multiple comparisons post-test in (**b–e**). Asterisks or p values above bars indicate significance compared to vehicle control, with ****, ***, ** and * indicating $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

Like DPV and TDF, MVC was shown to not significantly induce expression of the reporter above basal levels at concentrations ranging from 0.01 nM–10 μM and did not significantly alter the transcriptional activity of DEX, MPA or LNG in COS-1 cells (Appendix **Fig. A2**). Experiments with MVC were performed in the COS-1 model system, prior to all other experiments. The use of COS-1 cells was discontinued in favor of using U2OS cells, which, unlike COS-1 cells, are of human origin, and therefore, better suited to the objectives of this study. Due to time constraints the experiments with MVC were not repeated in U2OS cells.

3.2 *DPV and TDF modulate the efficacy, but not potency of a GR agonist*

Although DPV and TDF may not activate the GR directly, they have the potential to modulate GC action, possibly through affecting one or more of the numerous proteins involved in GR signaling. Using promoter-reporter assays, the effect of 1 μM DPV (**Fig. 3.2a**) or TDF (**Fig. 3.2b**) on the efficacy and potency (EC_{50}) of DEX was investigated. TDF significantly increased the efficacy of DEX transcriptional activity via the GR by 33% (**Fig. 3.2c**); DPV had a similar non-statistically significant effect ($p=0.0531$), increasing DEX transcriptional activity by 25% (**Fig. 3.2c**). DPV and TDF did not significantly alter the EC_{50} of DEX (**Fig. 3.2d** & **e**). These effects were not detectable in Fig. 3.1 for 1 μM ARV, where the effects of only 100 nM GR ligand were investigated. Taken together, although DPV and TDF do not independently transactivate the GR (**Fig. 3.1**), they modulate GC transcriptional activity in these reporter assays (**Fig. 3.2**) via the GR in U2OS cells.

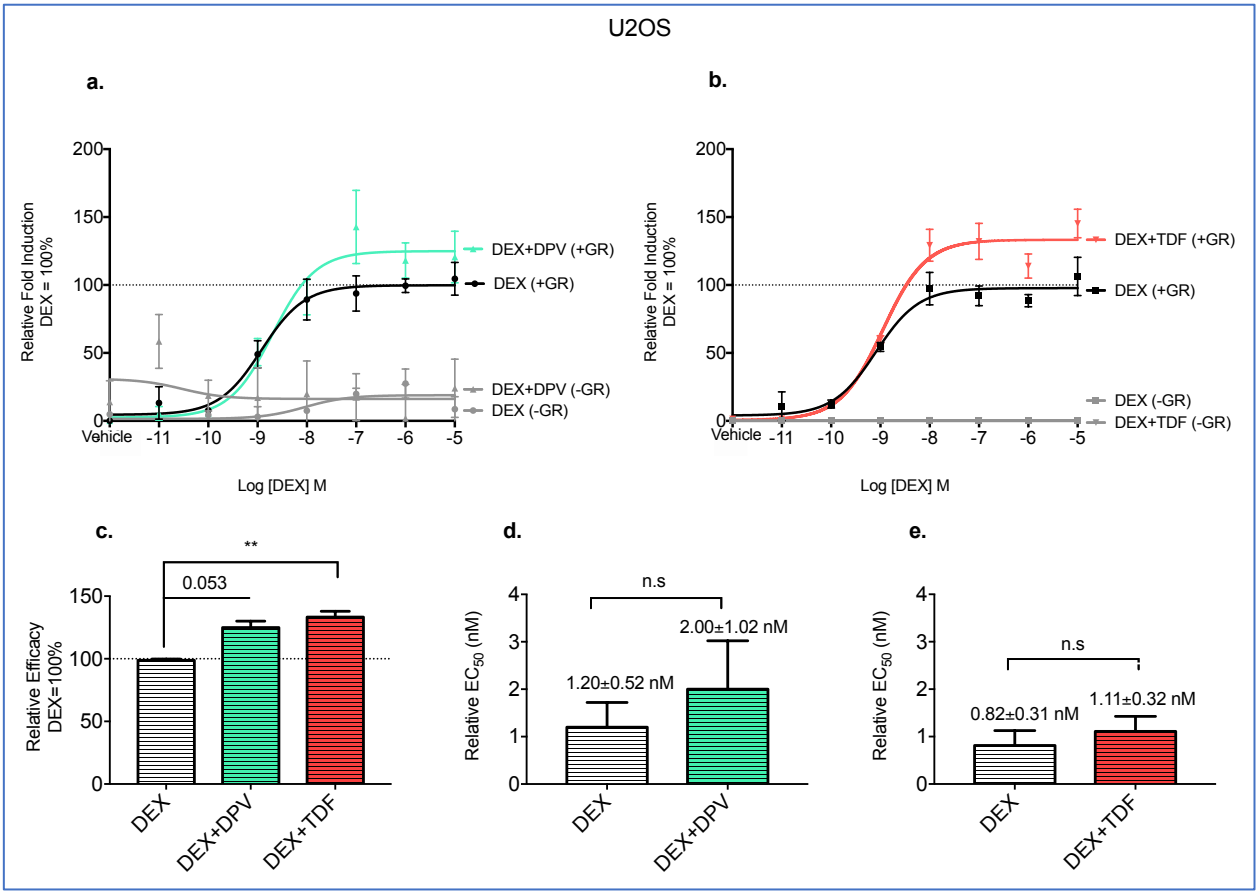

Figure 3.2: **DPV and TDF increase the transcriptional efficacy of DEX via the GR.** U2OS cells were co-transfected with the GR expression vector (+GR) or its empty vector control (-GR), and the GRE-luciferase reporter construct. Subsequently, cells were treated with increasing concentrations of DEX, in the presence or absence of 1 μM DPV (**a**) or TDF (**b**), or vehicle (0.1% *v*/*v* EtOH and DMSO). Luciferase activity was normalized to protein content per well, as determined by a Bradford assay. The maximal value of DEX was set to 100% to obtain relative fold induction. Relative potencies of the resulting dose-response curves are shown in (**c**), and relative EC_{50} values in (**d**) and (**e**), where the EC_{50} and its error are indicated above relevant bars. Data are plotted as mean ± SEM, and are the pooled results of three or more independent experiments, each performed in triplicate. Statistical significance for EC_{50} values and efficacies were determined using unpaired t tests. ** denotes $p< 0.01$ and n.s (not significant) $p>0.05$.

3.3 *DPV and TDF do not alter U2OS cell viability*

Alterations in cell viability may result in changes in the transcriptional efficacy of GCs via the GR, as well as GC-induced mRNA expression. The capacity of DPV and TDF to affect cell viability alone, and in combination with DEX (**Fig. 3.3a**), CORT (**Fig. 3.3b**) or MPA (**Fig. 3.3c**) was investigated. Cells were treated with DEX or MPA at concentrations ranging from 0.01 nM–10 μM and CORT at concentrations ranging from 10 nM–1 μM, with or without the addition of 1 μM DPV or TDF. At the concentrations tested, DEX, MPA and CORT did not significantly alter the viability of U2OS cells compared to vehicle. The addition of DPV or TDF had no significant

effect. These results suggest that the ARV-induced increases in DEX transcriptional efficacy via the GR observed in **Fig 3.2** were not due to differential effects on cell viability.

To this point DPV and TDF have been shown to not independently transcriptionally activate the GR, or alter U2OS cell viability, alone or in the presence of GCs. However, DPV and TDF modulate the transcriptional efficacy of DEX on a GRE-reporter gene in these cells.

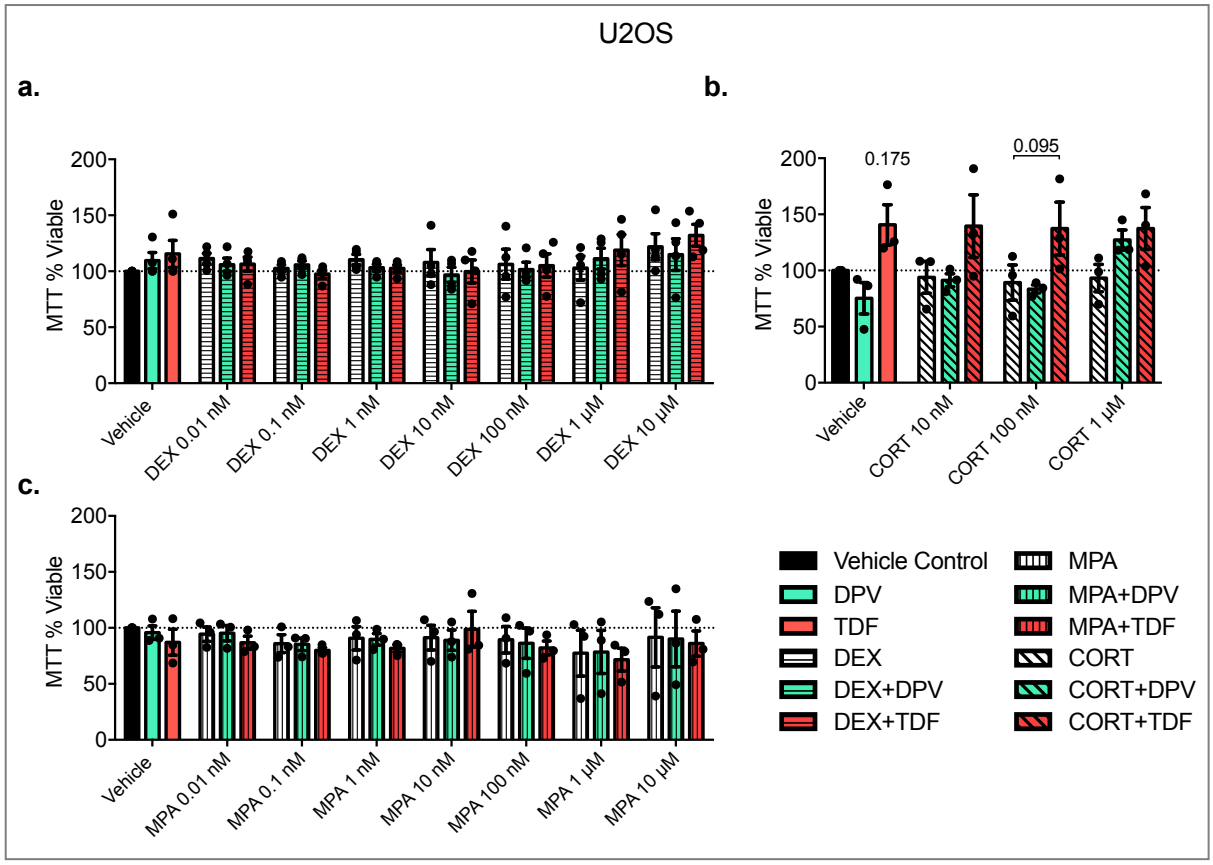

Figure 3.3: **DPV and TDF, in the absence and presence of GR ligands, do not significantly alter the viability of U2OS cells**. U2OS cells were transfected with the GR expression vector and subsequently, cells were treated with DEX (**a**), CORT (**b**) or MPA (**c**), or vehicle (0.1% *v/v* EtOH and DMSO), in the absence or presence of 1 µM DPV or TDF for 24 hours as indicated. Cell viability was determined using MTT assays. Data are plotted relative to vehicle control (black bar), which was set to 100%. Data are plotted as mean ± SEM and are the pooled results of three or more independent experiments, each performed in quadruplicate. Statistical significance was determined using a two-way ANOVA with a Tukey's multiple comparisons post-test. No statistically significant differences were observed, but p values above bars indicate significance compared to vehicle control (black bar), unless otherwise indicated with lines.

3.4 *TDF modulates DEX-induced mRNA expression in U2OS cells*

Following observations that DPV and TDF increased the transcriptional efficacy of DEX on a GRE-reporter gene, their capacity to modulate GR-regulated mRNA levels of immune function genes was assessed (**Fig. 3.4**). Prior to cDNA synthesis, the integrity of all isolated RNA was

assessed by denaturing formaldehyde gel electrophoresis, as shown in the insert in (**Fig. 3.4a**), where distinct 28S and 18S RNA bands are visible. DEX, at concentrations of 1 μM and 10 μM, significantly induced GILZ mRNA expression by approximately 7-fold relative to vehicle. Contrary to the reporter gene results, the addition of 1 μM TDF, but not DPV significantly decreased DEX-induced GILZ maximal mRNA expression by approximately 5-fold (**Fig. 3.4a & b**). There were no significant differences in the EC_{50} observed. In cells transfected with the empty vector control, (0.01 nM–1 μM) DEX did not induce GILZ mRNA expression (see Appendix **Fig. A3**). Although both DPV and TDF increased the expression of CORT-induced GILZ mRNA at some of the concentrations tested, the effect was not statistically significant. The overall maximal efficacy of CORT decreased slightly, but the effect was not statistically significant (p=0.415) in the presence of TDF (**Fig. 3.4d**). Neither ARV significantly altered MPA-induced GILZ maximal mRNA expression (**Fig. 3.4e**). The mRNA expression of IκB-α was also assessed. As with GILZ, IκB-α expression is known to be upregulated by GCs (Deroo and Archer, 2001). However, in these experiments DEX did not significantly induce IκB-α mRNA expression above basal levels after 24 hours, and the addition of 1 μM DPV or TDF did not alter expression (**Fig. 3.4c**). Given the large errors in the dose-response analysis (Fig. **3.4a, d** & **e)**, the observed differential effects of TDF on DEX-, CORT- and MPA-induced GILZ mRNA expression were further investigated. Cells were treated with 1 μM DEX, CORT or MPA, in the absence or presence of 1 μM TDF in parallel (**Fig. 3.4f**). Similar to the results obtained earlier (**Fig. 3.4a**), DEX significantly induced GILZ mRNA expression, by approximately 5-fold relative to vehicle and TDF decreased DEX-induced GILZ mRNA expression, but this effect was not statistically significant. However, the degree of change in DEX-induced GILZ mRNA expression in the presence of TDF was less than previously observed and not significant statistically (p=0.089). CORT and MPA induced non-statistically significant increases in GILZ mRNA expression by approximately 2.5-fold and 4-fold, respectively (**Fig. 3.4f**), and TDF decreased MPA and CORT-induced GILZ mRNA expression, but these effects were not statistically significant.

While both DPV and TDF increase the transcriptional efficacy of DEX in reporter assays in U2OS cells (**Fig. 3.2**), the ARVs have differential effects on DEX-induced GR-regulated mRNA expression. TDF, but not DPV, significantly decreases DEX-induced endogenous GILZ mRNA expression, and has similar apparent effects on CORT- and MPA-induced GILZ mRNA expression in U2OS cells. However, the effect of TDF on (1 μM) DEX-induced GILZ mRNA expression proved to be highly variable (**Fig. 3.4f**). These ARV effects are unlikely to be due to differential effects on cell viability (**Fig. 3.3**).

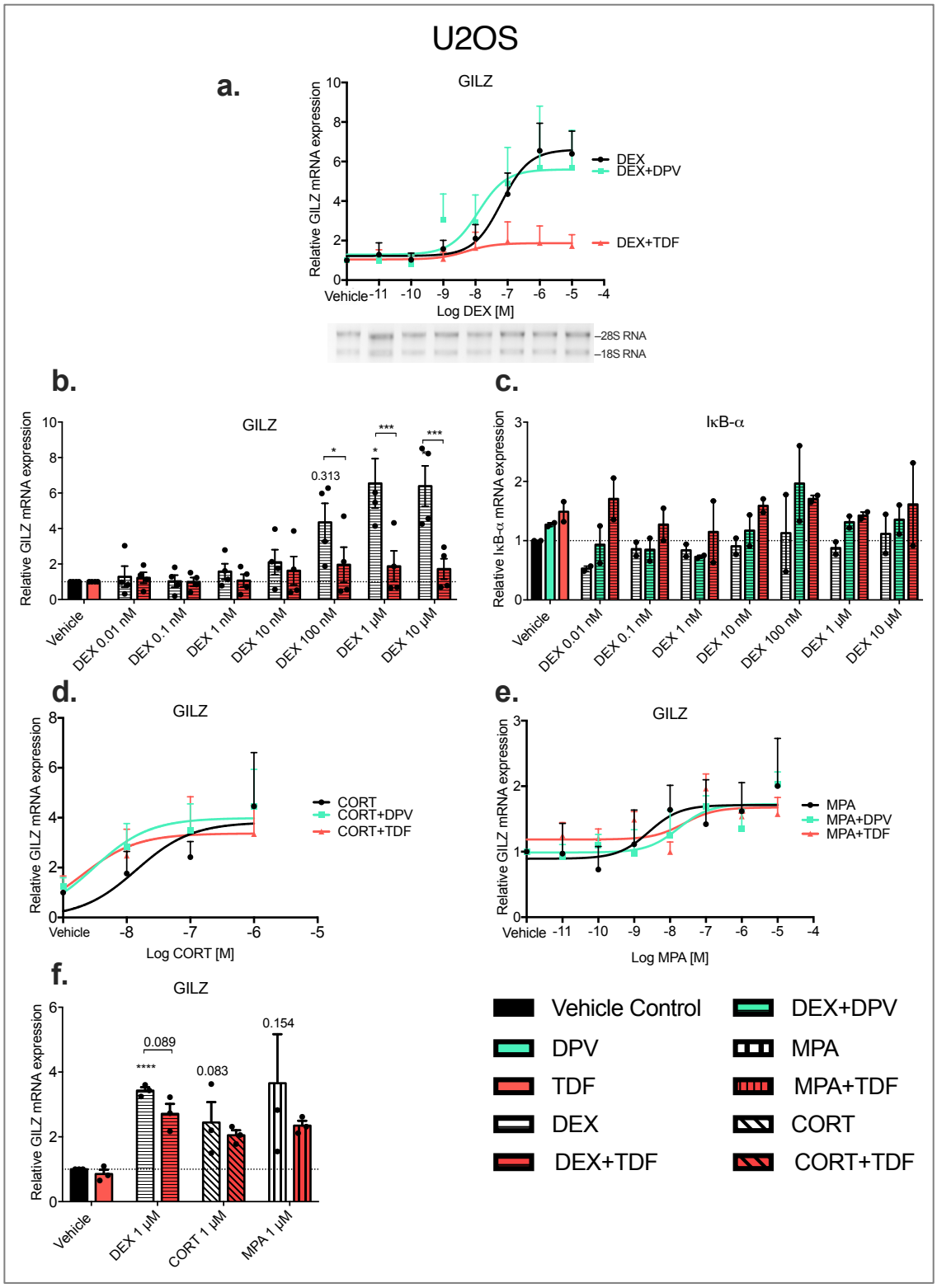

Figure 3.4: **TDF alters DEX-induced GILZ mRNA levels in U2OS cells.** U2OS cells were transfected with the GR expression vector. Subsequently, cells were treated with increasing concentrations of DEX (**a**–**c**), CORT (**d**) or MPA (**e**), or vehicle (0.1% *v*/*v* EtOH and DMSO) in the absence or presence of 1 µM DPV or TDF for 24 hours as indicated. RNA was isolated, and its integrity assessed by denaturing formaldahyde gel electrophoresis as shown in the insert (bottom) in (**a**). cDNA was synthesized and the expression of GILZ and IκB-α mRNA was determined and normalized to GAPDH mRNA levels. Data are plotted as mean ± SEM and are the pooled results of three or more independent experiments, with the

exception of (**c**) and (**e**), which are the pooled results of two independent experiments. All experiments were performed in triplicate. A subset of the data shown in (**a**) is also shown in (**b**), plotted as bar graphs with statistical comparisons. Relative fold change in expression was determined by setting vehicle control (for steroid without ARV) to 1. Statistical significance was determined using a two-way ANOVA with a Tukey's multiple comparisons post-test in (**a-e**) and unpaired t-tests in (**f**). Asterisks or p values above bars indicate significance compared to vehicle control (black bar), unless otherwise indicated with lines. ****, ***, ** and * indicate $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

3.5. *The effect of DPV and TDF on GR protein levels and GR phosphorylation*

To gain mechanistic insight into the observed effects of DPV and TDF on the transcriptional activity of DEX (**Fig. 3.2**) and DEX-induced mRNA levels (**Fig. 3.4**), their effects on GR protein expression and phosphorylation at Ser226 (pSer226-GR) in U2OS cells were assessed by western blotting (**Fig. 3.5**). Ser226 is hyperphosphorylated in the presence of GCs and has been shown to enhance nuclear export, (Itoh *et al.*, 2002) and is thus associated with blunting GR responses (Kino *et al.*, 2007). As expected and previously observed in (**Fig. 3.1a**) DEX, at concentrations of 100 nM and 1 μM induced reductions in GR protein levels of approximately 50%, although these effects were not statistically significant (**Fig. 3.5b**). DEX, at concentrations of 100 nM and 1 μM, induced 200% and 300 % non-statistically significant increases, respectively, in pSer226-GR levels (**Fig. 3.5c**). The addition of DPV and TDF resulted in non-statistically significant increases in (1 μM) DEX-induced phosphorylation of the GR at Ser226 by approximately 19% and 68%, respectively (**Fig. 3.5c**). Interestingly, at a concentration of 1 μM DEX, GR levels were approximately 50% lower in the presence of TDF, but this effect was not statistically significant (**Fig. 3.5b**). Individual western blots are shown and quantified in the Appendix (**Fig. A4-A6**).

No statistically significant differences were obtained between GR levels or phosphorylation at Ser226 for different conditions in these pooled data. Given the large number of variables, relatively small apparent differences and inherent technical error in the quantification, it was not possible to establish whether the apparent differences observed were statistically significant or not, without performing more experiments with fewer variables.

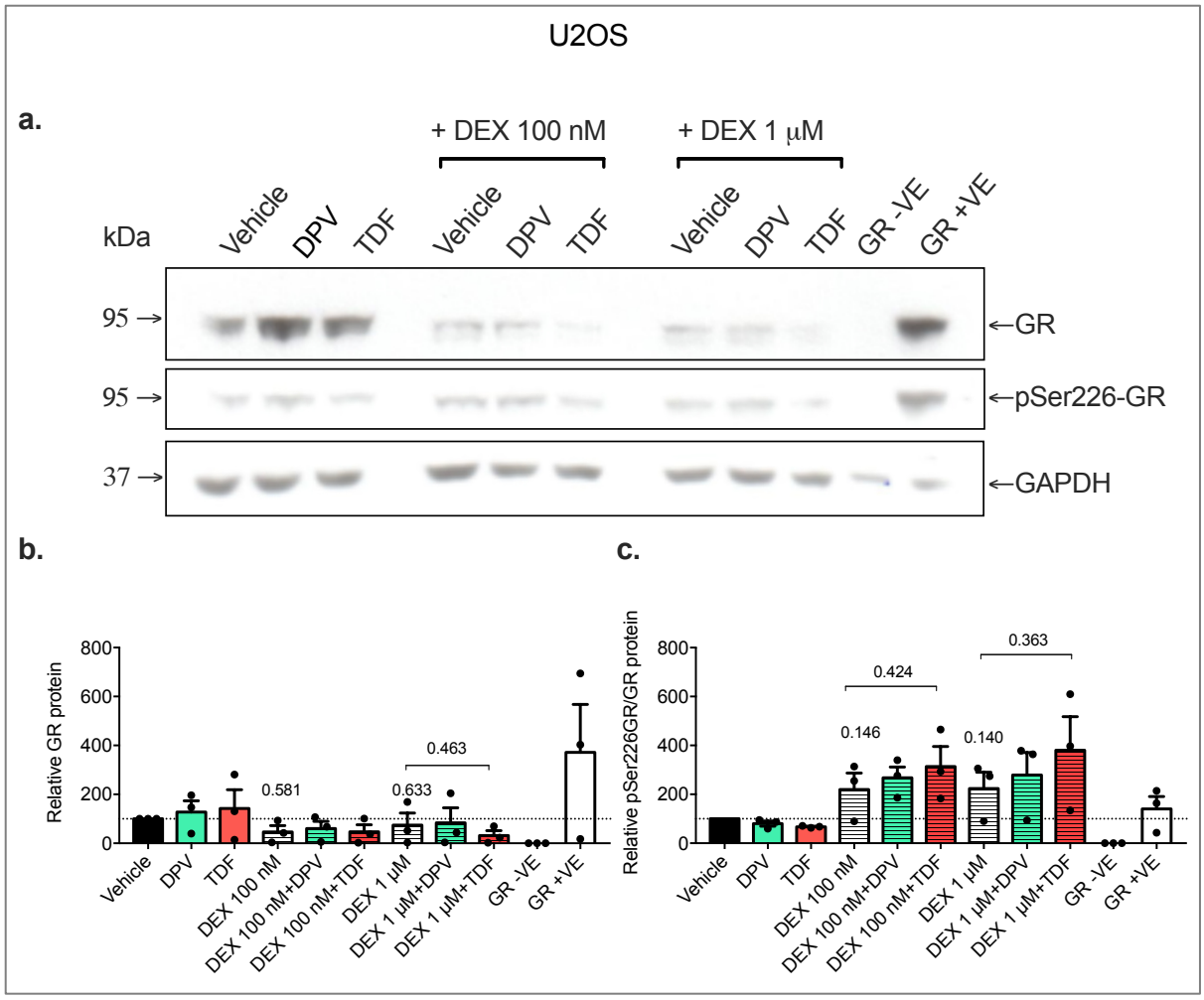

Figure 3.5. **DPV and TDF effect on GR protein levels and GR phosphorylation in U2OS cells.** U2OS cells were transfected with the GR expression vector and subsequently stimulated with 1 μM DPV or TDF in the absence or presence of 100 nM DEX, 1 μM DEX or vehicle (0.1% *v*/*v* EtOH and DMSO) for 24 hours. Equal volumes of cell lysate were analysed by western blotting with GR and pSer226-GR specific antibodies, with GAPDH as a loading control. A representative western blot is shown in (**a**). Quantification of GR protein levels is shown in (**b**) and relative pSer226-GR levels in (**c**). Data are plotted as mean ± SEM and are the pooled results of three independent experiments. Relative fold change in expression was determined by setting vehicle control (black bar) to 100%. Statistical significance was determined using unpaired t-tests. No statistically significant differences were observed, but p values above bars indicate significance compared to vehicle control, unless otherwise indicated with lines.

3.6 *DPV, TDF and MVC modulate GR-regulated mRNA expression in PBMCs in a donor-specific manner*

To further assess the ability of DPV, TDF, as well as MVC to modulate the expression of immunomodulatory genes, PBMCs were treated with 1 μM DPV, TDF or MVC, alone or in combination with 100 nM DEX for 48 hours (previous experiments with steroids and progestins in the authors laboratory have shown 48 hours is reasonable to invoke robust inflammatory responses in PBMCs) (**Fig. 3.6**). In PBMCs from the majority of donors, DPV, TDF and MVC

significantly decreased the expression of anti-inflammatory GILZ mRNA (**Fig. 3.6a**). DEX induced GILZ mRNA expression by approximately 6-fold, but this effect was not statistically significant (p=0.050), which remained unchanged in the presence of either ARV in the majority of donors. DEX significantly repressed IL-6 mRNA expression by approximately 2-fold relative to vehicle (**Fig. 3.6b**), but did not significantly alter IL-8 (**Fig. 3.6c**) or IFN-γ (**Fig. 3.6d**) mRNA levels. DPV, TDF and MVC had no overall significant effects on IL-6, (**Fig. 3.6b**), IL-8 (**Fig. 3.6c**) or IFN-γ (**Fig. 3.6d**) mRNA expression when examining pooled data. However, apparent donor-specific effects on the mRNA levels of all genes were observed, and are shown in Appendix **Fig. A7–A10**. The addition of DEX significantly reversed the apparent ARV-induced increases in IL-6 and IL-8 mRNA expression (**Fig. 3.6b & c**). As in U2OS cells, DEX and the ARVs had no significant effect on IκB-α mRNA levels (**Fig. 3.6e**). DPV and TDF significantly decreased the viability of PBMCs by 5% and 6% relative to vehicle, respectively (**Fig. 3.6f**). Treatment with DEX significantly reduced the viability of PBMCs by approximately 20% relative to vehicle, which was unchanged in the presence of MVC, DPV or TDF (**Fig. 3.6f**).

The results in **Fig 3.6** suggest there are large inter-donor differences in responses, which was investigated by subgroup analysis (Appendix **Fig. A7–A10)**. Whether the effects in these subgroups are statistically significant was not investigated since they were grouped post the accumulation of the data set. The groups are shown to illustrate the possibility that there may be donor-specific effects that are masked by pooling all the data. However further experiments are needed to investigate the statistical significance of these effects, by perhaps selecting donors beforehand based on some exclusion criteria. MVC, DPV and TDF decreased GILZ mRNA levels by 2-fold or more in PBMCs from seven of the nine donors tested (Appendix **Fig. A7a–c**). MVC decreased IL-6 mRNA levels by 2-fold or more in PBMCs from one donor, while DPV and TDF did so in PBMCs from two donors (Appendix **Fig. A8a–c**). MVC increased IL-6 mRNA expression by 2-fold or greater in PBMCs from four donors, and DPV and TDF had the same effect in PBMCs from two donors (Appendix **Fig. A8g–i**). MVC and TDF decreased IL-8 mRNA expression (by 2-fold or greater) in PBMCs from two donors, and DPV did so in PBMCs from three donors (Appendix **Fig. A9a–c**). MVC and TDF increased IL-8 mRNA levels by more than 2-fold in PBMCs from four donors, and DPV had a similar effect in PBMCs from three donors (Appendix **Fig. A10g–i**). MVC and TDF reduced IFN-γ mRNA expression by 2-fold or more in PBMCs from one donor, and DPV did so in PBMCs from two donors (Appendix **Fig. A10a–c**). MVC increased IFN-γ mRNA expression by 2-fold or more in PBMCs from four donors, and DPV and TDF had a similar effect in PBMCSs from three donors (Appendix **Fig. A10g–i**).

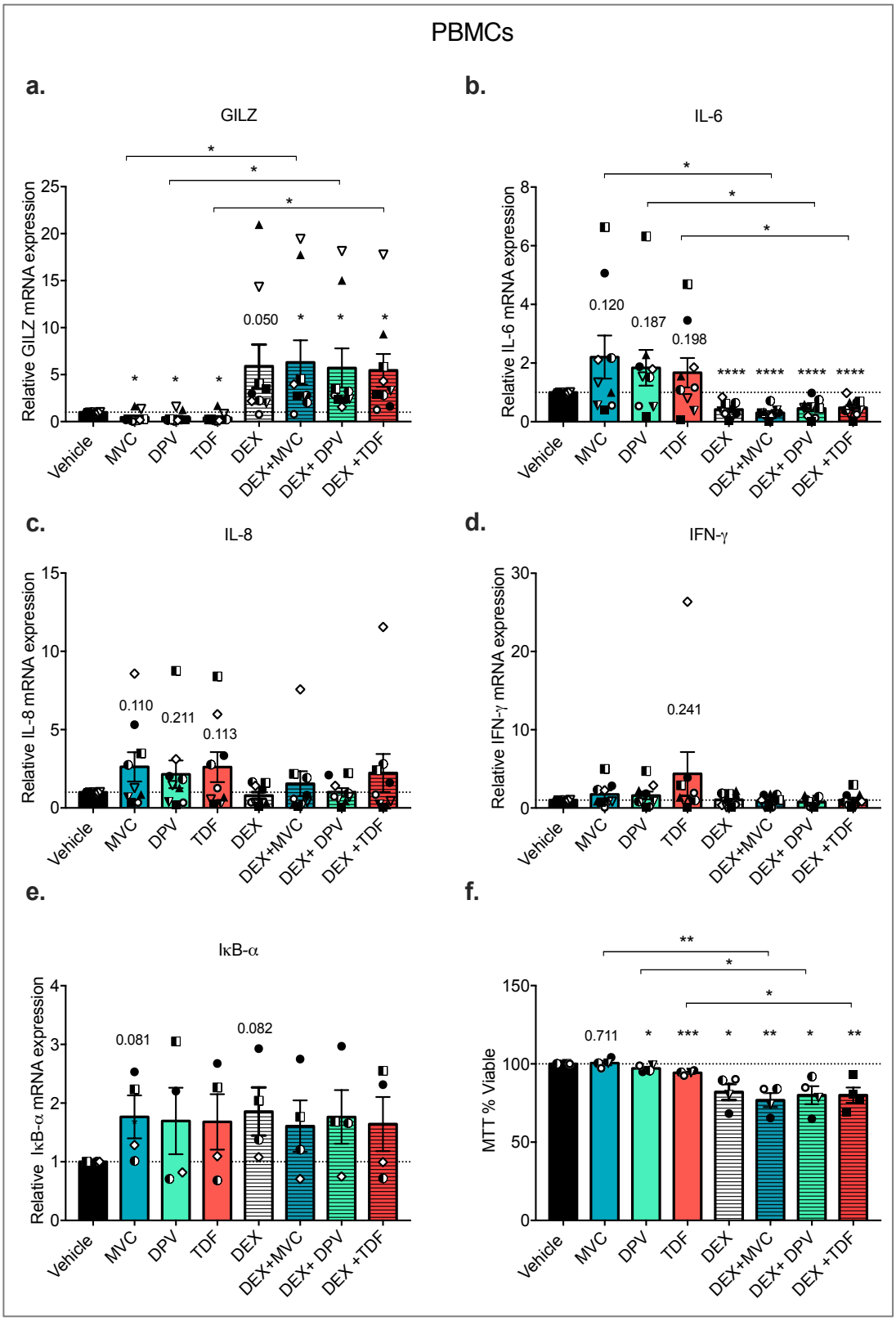

Figure 3.6: **DPV, TDF and MVC selectively alter immunomodulatory gene mRNA levels in PBMCs.** PBMCs were stimulated with or 1 μM MVC, DPV or TDF, in the absence or presence of 100 nM DEX, or vehicle (0.1% *v*/*v* EtOH and DMSO) for 48 hours. Subsequently, relative changes in GILZ (**a**) IL-6 (**b**) IL-8 (**c**) IFN-γ (**d**) and IκB-α (**e**) mRNA expression were determined by real time qPCR, and normalized to GAPDH mRNA levels. The effects of treatments on cell viability were determined using an MTT assay (**f**). Relative fold change in expression was determined by setting vehicle control (black bar) to

1 (**a**–**e**) or 100% (**f**). Pooled results of nine donors are shown. Data are plotted as mean ± SEM. Individual donors are depicted with specific black symbols. Statistical significance was determined using unpaired t-tests, ****, ***, ** and * indicate $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

3.7 *DEX and MPA do not alter HIV-1 inhibitory effects of DPV or TDF*

Lastly, the potential of progestins and GCs to modulate the anti-HIV-1 effects of DPV and TDF was examined (**Fig. 3.7**). DPV and TDF at concentrations of 1 μM completely inhibited the replication of HIV-$1_{BaL\text{-}Renilla}$ in PBMCs from two and three donors, respectively. In PBMCs from most donors, the addition of DEX, MPA and LNG had no significant effect on the efficacy of DPV and TDF to inhibit HIV-$1_{BaL\text{-}Renilla}$ replication. However, in PBMCs from one donor, the addition of LNG apparently reduced the efficacy of TDF to inhibit HIV-1 replication, by approximately 50% relative to control. As such, TDF in the presence of LNG did not significantly inhibit infection relative to control for pooled results from three donors. In another donor, DPV alone repressed infection by 80%, and completely when in the presence of DEX, MPA and LNG. It may be that the steroids had no effect on the anti-HIV activity of the ARVs as they were used at a relatively high concentration, and any modulatory effect of the steroid may have been overwhelmed by the ARV. However, in the authors hands, lower doses of the ARVs (1 nM and 100 nM) were not significantly protective against HIV-1 infection in PBMCs (data not shown), thus a higher concentration was used.

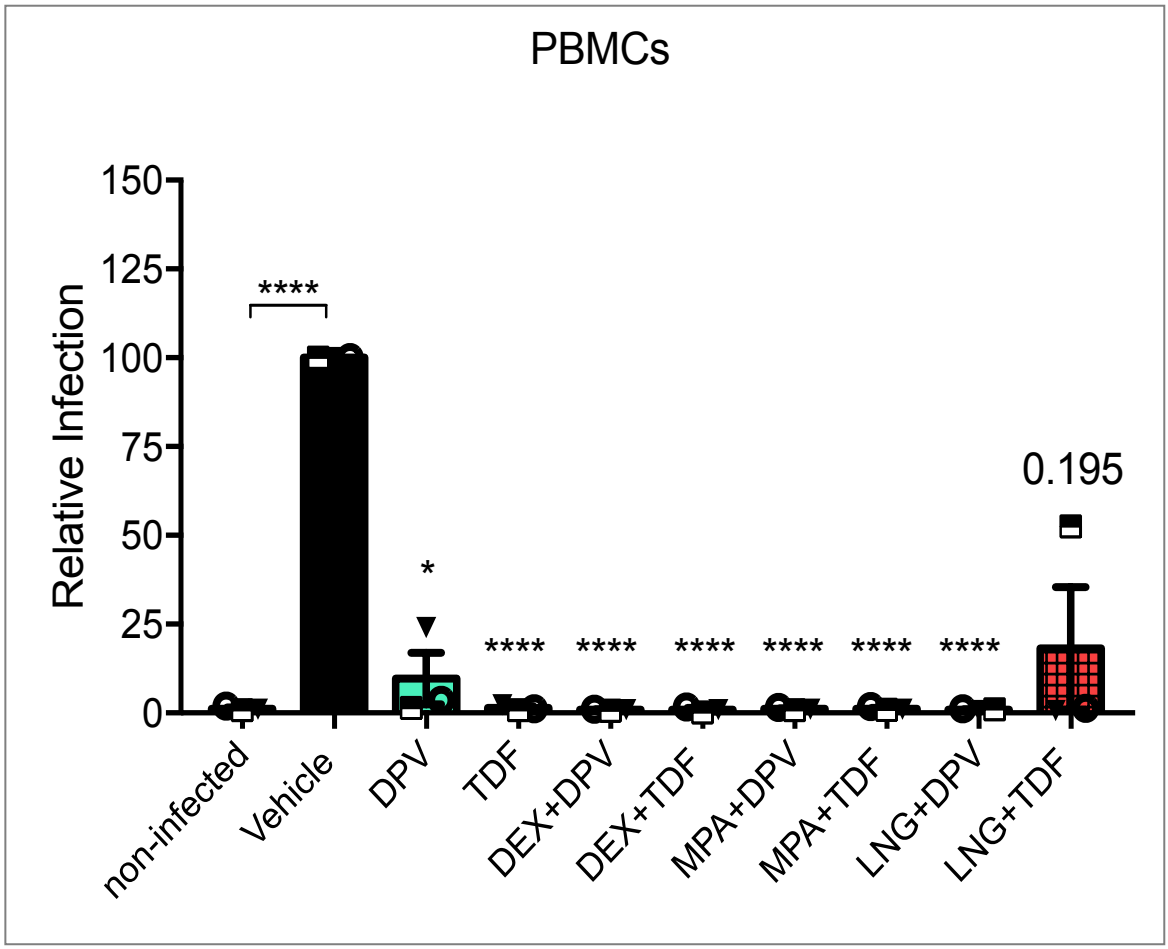

Figure 3.7: **DEX, MPA and LNG do not alter the HIV-1 inhibitory effects of DPV or TDF.** PBMCs were stimulated with 1 μM DPV or TDF, in the absence or presence of 100 nM DEX, MPA or LNG, or vehicle (0.1% *v*/*v* EtOH and DMSO) for 48 hours. Thereafter, cells were infected with HIV-$1_{BaL\text{-}Renilla}$ which was allowed to propagate for 5 days. Infection was determined by measuring luminescence and normalized to cell viability, as determined by an MTT assay. Pooled results from 3 donors in quadruplicate are shown. Relative infection was determined by setting vehicle control to 100%. Data are plotted as mean ± SEM. Individual donors are depicted with specific black symbols. Statistical significance was determined using a one-way ANOVA with a Tukey's multiple comparisons post-test. Asterisks or p values above bars indicate significance compared to vehicle control (black bar), unless otherwise indicated with lines. ****, ***, ** and * indicate $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

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CHAPTER 4
DISCUSSION

With the development of DPV and TDF PrEP for the prevention of HIV-1 acquisition, it is important to characterize the immunomodulatory effects of these ARVs. Increased access to HAART, as well as HCs, has led to an increase in their concurrent use. Moreover, this is likely to increase, owing to the development of MPTS; ARV and progestin combination products for the simultaneous prevention of HIV-1 acquisition and unintended pregnancy. Intracellularly, ARVs are in the presence of GCs during times of stress or GC therapy. As such, it is important to investigate possible reciprocal modulation of ARV and steroid receptor intracellular actions, which may alter their efficacy. This study evaluated the capacity of DPV and TDF to transcriptionally activate the GR, modulate the efficacy and potency of a GR agonist via the GR, affect mRNA levels of GR target genes, as well as the effects of these ARVs on GR expression levels and GR phosphorylation in an *in vitro* cell line model. Their effects on mRNA levels of immunomodulatory genes were assessed in PBMCs, as were the effects of DEX, MPA and LNG on the HIV-1 inhibitory effects of DPV and TDF.

This study showed DPV and TDF can modulate the reporter gene transcriptional efficacy of DEX via the GR in the model system used, as well as DEX-induced changes in mRNA levels of immunoregulatory genes, at physiologically relevant concentrations (see **Table 1.2 & 1.3** for ARV and GC serum concentrations). In the U2OS cell line model, TDF significantly decreased DEX-induced anti-inflammatory GILZ mRNA expression and to decrease CORT- and MPA-induced GILZ mRNA expression. In the presence of TDF, an apparent increase in DEX-induced phosphorylation of the GR at Ser226 was observed in U2OS cells. In PBMCs, the ARVs decreased basal GILZ mRNA expression, and had varied, donor-specific apparent effects on the expression of IL-6, IL-8 and IFN-γ. This study showed no effect of DEX, LNG or MPA on the HIV-1 inhibitory effects of DPV or TDF in PBMCs from the majority of donors tested.

4.1 *Effects of DPV and TDF on GR-mediated transcriptional activity and mRNA expression*

A variety of signals, notably TNF and GnRH, have been shown to transcriptionally activate the GR, in the absence of GCs (Kotitschke *et al.*, 2009; Verhoog *et al.*, 2011; Hapgood *et al.*, 2016).

The ability of DPV and TDF to transcriptionally activate the GR was assessed in U2OS cells exogenously expressing the GR, using GRE-luciferase reporter assays (**Fig. 3.1**). DPV and TDF did not activate the GR at any of the concentrations tested, alone or in the presence of 100 nM DEX (**Fig. 3.1**), LNG or MPA (Appendix **Fig. A1**). These results are consistent with a previous study for TDF (Svärd *et al.*, 2014), while the result for DPV is novel. Svärd *et al.* found that a panel of 26 ARVs, which included TFV but not DPV, had no LBD interactions with the GR, or ability to induce GR transactivation *in vitro* (Svärd *et al.*, 2014). Theoretically, it is unlikely that TDF and DPV can bind to the GR LBD, given that the GR LBD predominantly binds to four-ring steroidal compounds (Schmit and Rousseau, 1979), dissimilar from the chemical structures of TDF or DPV (see **Fig. 1.2**). The results of the present study suggest DPV and TDF, at physiologically relevant concentrations, do not transactivate gene expression via the unliganded GR *in vitro* or *in vivo*. Therefore, ligand-independent activation of the GR by these ARVs is unlikely to be a mechanism contributing to their effects on immunomodulatory gene expression in U2OS cells and PBMCs in this study, or responsible for the adverse side effects observed in users of the ARVs.

The capacity of DPV and TDF to alter the transcriptional activity of DEX via the GR was also assessed using reporter assays (**Fig. 3.2**). TDF significantly increased the efficacy of DEX via the GR by approximately 33%, and DPV had a similar apparent effect. This is the first study to show that ARVs affect GC-induced transcriptional activity via the GR. In this study, the EC_{50} of DEX, which was approximately 1 nM, was not altered in the presence of DPV or TDF (**Fig. 3.2d & e**). The EC_{50} is dependent on the cell type, promoter and receptor concentrations, ligand affinity for the receptor and, as is apparent in **Fig. 3.2d & e**, is variable between experiments (Robertson *et al.*, 2013; Hapgood *et al.*, 2014). The EC_{50} of DEX in this experiment is similar to reported EC_{50} values for the GR of 1.66 nM and 1.03 nM, determined by others in U2OS cells using reporter genes (Sedlák *et al.* 2011). The efficacy is influenced by the affinity of the ligand for the receptor, receptor concentrations and the cell specific relative concentrations of co-regulators (Ronacher *et al.*, 2009; Simons and Chow, 2012). Growth factors, cytokines and LABAs have been shown to prime the unliganded GR, increasing its sensitivity to GCs, but not transcriptionally activating it, via mechanisms involving altered GR and/or cofactor recruitment or binding to the promoters of genes, altering the activity or levels of signaling proteins in the GR pathway, including kinases and cofactors, and changes in site-specific phosphorylation of the GR (Hapgood *et al.*, 2016). It is possible DPV and TDF sensitize GR signaling via similar mechanisms in U2OS cells, although this remains to be determined. The present study found DPV and TDF had no significant effect on GR protein levels in U2OS cells, alone, or in the presence of DEX (**Fig. 3.5**). Therefore ARV-

induced alterations in GR concentrations are unlikely to be a mechanism through which the ARVs modulate GC activity in U2OS cells. An obvious mechanism through which ARVs may alter GR-mediated responses is through altering cell viability. This study demonstrated that 1 μM DPV and TDF, alone and in the presence of DEX, CORT and MPA, did not significantly alter the cell viability of U2OS cells exogenously expressing the GR, after 24 hours (**Fig. 3.3**). Moreover, the steroids did not significantly alter the viability of these cells either. Therefore, the effects of DPV and TDF on cell viability cannot account for their effects on steroid-induced responses via the GR.

Contrary to the results of the promoter-reporter assays, TDF significantly decreased DEX-induced GILZ mRNA expression by approximately 5-fold, whereas DPV had no significant effect (**Fig 3.4a & b**). Several factors may explain the discrepancy between the ARVs effects on DEX-induced reporter gene transcription, and DEX-induced endogenous GILZ mRNA expression. Inherent to promoter-reporter assays is the absence of adjacent condensed regions of chromatin and epigenetic modifications, which regulate transcriptional responses through influencing the three-dimensional spatial arrangement of the linear genome in the nucleus, in this way dictating promoter and enhancer element accessibility to transcription factors and co-regulators (Burd and Archer, 2013). Chromatin also dictates GR genomic interactions with distal enhancer elements (which are absent in the context of reporter assays) for example, the GR-regulated genes Ciz1 and Lcn2 are regulated cell-specifically through a chromatin loop which spans 30 kb (Hakim *et al.*, 2011). Besides, hundreds of promoter-reporter plasmids may be transfected into a cell, but transcription factors and co-regulators are typically present at low concentrations (Smith and Hager, 1997; Karimi *et al.*, 2009). As such, only a small proportion of the reporter genes entering a cell receive all necessary transcription factors, and the remaining promoter-reporter constructs may be atypically transcribed, resulting in transcriptional effects which differ from those of endogenous genes (Smith and Hager, 1997; Karimi *et al.*, 2009).

It should also be noted that regulatory elements present on the promoter-reporter plasmid used in this thesis differ substantially from those of the endogenous GILZ gene. On the reporter plasmid, two GREs from the rat TAT gene are cloned upstream of the luciferase gene, whereas the endogenous human GILZ gene promoter includes five GREs, three Forkhead box class O3 binding sites, as well as putative signal transducer and activator of transcription 6, NFAT and c-myc response elements (Asselin-Labat *et al.*, 2004). These represent potential targets through which ARVs may alter GC-induced gene expression. It is therefore conceivable that the GR exists

in distinct transcription initiation complexes endogenously and on the promoter-reporter plasmid, which differentially influence their interactions with co-regulators, ultimately resulting in differential responses. Apart from control at the level of transcription initiation, mRNA post-transcriptional processing contributes to gene regulation (see **Section 1.6.2**) (Kumar. *et al.*, 2014). If post-transcriptional regulation occurs differentially between luciferase and GILZ mRNA, this may contribute to the differences observed in the present study.

Although TDF induced a significant decrease in DEX-induced GILZ mRNA expression, this effect was not replicated on the other GC-induced gene, IκB-α, investigated in this study (**Fig. 3.4c**). This may be due to differences in the promoters of the two genes. DEX did not significantly alter IκB-α mRNA expression compared to basal levels after 24 hours; it was therefore not possible to determine whether the ARVs modulate GC-induced expression of this gene. As such, it is not clear from the results of the current study, if TDF has gene-specific effects on DEX-induced mRNA expression in U2OS cells. The presence of TDF also appeared to decrease CORT- and MPA-induced GILZ mRNA expression, but this result was not statistically significant (**Fig. 3.4d & e**). Parallel comparison of the effects of TDF on DEX-, CORT- and MPA-induced GILZ mRNA expression also showed apparent decreases (**Fig. 3.4f**). Given that mRNA expression induced by more efficacious agonists was repressed to a greater degree (**Fig. 3.2a, d &e**), one may speculate that TDF-induced repression of GR-mediated GILZ mRNA expression is dependent on the efficacy of the GR agonist. Given that cells vary in their expression of co-regulators, which influence ligand efficacy (Ronacher *et al.*, 2009), the effects of ARVs on GC activity via the GR may also be dependent on cell type. Findings that TDF did not significantly alter DEX-induced GILZ mRNA expression in PBMCs in the present study (**Fig. 3.6a**) are consistent with this idea.

TDF has been reported to reduce the expression of genes involved in the MAPK signaling pathway in human primary osteocytes (Grigsby *et al.*, 2010; Espocito *et al.*, 2015). Similarly, Hladik *et al.* showed some genes involved in the MAPK signaling pathway were downregulated in rectal biopsies in the presence of a 1% TFV gel (Hladik *et al., 2*015). MAPK is known to induce activation of the GR through site-specific phosphorylation (Nader *et al.*, 2010). If TDF has similar effects on MAPK signaling in U2OS cells, and reduces GR activation, it is a possible mechanism through which it may decrease GC-induced GILZ expression. Alteration of GC-induced gene expression through modulating co-regulatory recruitment has previously been described (see **Section 1.6.6**). It is conceivable DPV or TDF could alter the function of one or more of the numerous co-regulators involved in GR signaling, thereby modulating GC responses as observed in the

present study. Intriguingly, in rectal biopsies, Hladik *et al.* showed a 1% TFV gel downregulated the expression of CREB and CBP, which are cofactors which mediate GR transactivation (Kamei *et al.*, 1996; Vo and Goodman, 2001; Hladik *et al.*, 2015). If TDF similarly downregulates CREB and CBP expression in U2OS cells, this may explain how it decreases DEX-induced GILZ mRNA expression. Moreover, because CBP has histone acetyltransferase activity, which is not a requirement for reporter gene transcription, TDF-induced downregulation of CBP would be consistent with findings in this study that TDF decreased DEX-induced GILZ mRNA expression, but not reporter gene transcription. A major mechanism through which the ARVs may modulate GC activity via the GR is through altering site-specific phosphorylation of the GR, which modulates all aspects of GR signaling, including cofactor recruitment, nuclear translocation, DNA binding, transactivation and transrepression (Hu *et al.*, 2013; Hapgood *et al.*, 2016; Kino, 2018).

4.2 *Effects of DPV and TDF on GR site-specific phosphorylation*

This study evaluated the effects of DPV and TDF on pSer226-GR levels in U2OS cells, alone, and in the presence of DEX (**Fig. 3.5**), and found that, analogous to their effects on DEX-induced GILZ mRNA expression, TDF induced a greater apparent increase in DEX-induced phosphorylation at Ser226 than did DPV. Phosphorylation at Ser226 modulates cofactor recruitment to DNA-bound GR (Kino *et al.*, 2007), and enhances GR nuclear export (Itoh *et al.*, 2002), and is thus associated with blunting GR responses (Kino *et al.*, 2007). Therefore, a mechanism by which TDF, but not DPV, may decrease GC-induced GILZ mRNA expression is through their differential effects on phosphorylation at Ser 226, resulting in nuclear export of the GR, and blunted GC-induced transcriptional responses. Phosphorylation at Ser226 has been shown to alter GR transcriptional activity in a promoter-selective manner, which may explain the varying effects of the ARVs on DEX-induced reporter gene transcription and GILZ mRNA expression (Avenant *et al.,* 2010b). The results of these experiments assessing GR phosphorylation are consistent with TDF decreasing DEX-induced GILZ mRNA expression, but not with DPV and TDF increasing DEX transcriptional activity on a reporter gene via the GR. These results are limited by their lack of statistical significance. Other instances of ligands desensitizing GR responses gene-specifically by altering site-specific phosphorylation of the GR have been described (refer to **Section 1.6.6**) (Hapgood *et al.*, 2016). It is tempting to speculate that DPV and TDF alter phosphorylation at other serine residues on the GR, which requires further investigation. Hindering the association of ARV effects on GR phosphorylation and their effects on GC-induced transcriptional activity or mRNA expression in the present study is the fact that

these experiments were not conducted in parallel, and samples assayed by western blotting were not transfected with the promoter-reporter construct, which potentially alters GR activity.

4.3 *Physiological implications of ARVs effects on GR activity*

GCs regulate all aspects of immune function, and modulate metabolic, cardiovascular and homeostatic physiological processes via the GR (Baschant and Tuckermann, 2010; Ramamoorthy and Cidlowski, 2013). This study did not investigate the effects of DPV and TDF on the efficacy and EC_{50} of CORT for reporter gene transcription via the GR but found that DPV and TDF increased the transcriptional efficacy of DEX at concentrations within the range it is used therapeutically (see **Table 1.3**). If one speculates the ARVs increase CORT transcriptional efficacy via the GR as with DEX, the results of this study have several implications, if translated *in vivo*. The reporter gene results suggest DPV and TDF may potentiate GC effects via the ubiquitous GR, particularly at times when circulating GC levels are high, for example, during times of psychological or physiological stress and GC therapy (Wenting-Van Wijk *et al.*, 1999, Dickerson and Kemeny, 2004). As such, systemically, DPV and TDF, used prophylactically or therapeutically, may have off-target adverse effects via the GR, modulating a wide array of GR-regulated physiological processes. Consistent with this premise, numerous clinical, animal and *in vitro* studies indicate the use of TDF is associated with decreases in BMD and increases in markers of bone turnover (Grant and Cotter, 2017). Likewise, GCs, increase the risk of osteoporosis and stimulate bone resorption, in part through inducing transactivation of the gene RANKL via the GR in osteoblasts (Canalis and Delany, 2002). In the present study, TDF potentiated the efficacy of a GC for reporter gene transcription in an osteoid cell line; if it similarly potentiates GC-induced RANKL expression in osteoblasts *in vivo,* this may be a mechanism which contributes to its adverse effects on bone.

However, the results of experiments using reporter genes were contrary to experiments evaluating endogenous mRNA transcription, which are more physiologically relevant, and suggest TDF but not DPV, can repress GC-induced mRNA expression via the GR. CORT and DEX concentrations used in this study were similar to those achieved during major-stress or GC-therapy (**Table 1.3**). If the results of the present study are translated *in vivo*, they imply TDF may have pronounced effects on DEX-induced gene expression in individuals using DEX therapeutically, perhaps decreasing its therapeutic efficacy by reducing its ability to induce anti-inflammatory gene expression. DEX and CORT differ substantially in their binding affinities (Ponec *et al.*, 1986), EC_{50}

values and efficacies via the GR in some cell types (Smit *et al.*, 2005; Ronacher *et al.*, 2009) Moreover, DEX has a half-life substantially longer than that of CORT (Peterson, 1959). For example, in PBMCs, when evaluating GILZ mRNA expression, the EC_{50} values and efficacies of DEX have been reported to be approximately 14-fold and 2-fold higher than for CORT, respectively (Smit *et al.*, 2005). However, because ligand- and promoter-selective responses are dependent on cell-specific co-regulator recruitment, in some cell types, CORT and DEX have similar efficacies and EC_{50} values (Ronacher *et al.*, 2009). Under such contexts, TDF may therefore also alter (exogenous or endogenous) CORT-induced anti-inflammatory gene expression *in vivo*. As such, the use of TDF prophylactically or therapeutically may result in a decreased ability to blunt immune responses and therefore increase inflammation and immune activation, the consequences of which are discussed in **Section 4.5**. One may speculate that TDF also has cell-specific effects on MPA-induced GR-mediated mRNA expression, although, given the supra-physiological doses of MPA (**Table 1.1**) at which TDF induced relatively small apparent decreases in GILZ mRNA expression in this study, TDF would probably not alter MPA-induced mRNA expression via the GR *in vivo*. It is possible TDF has more pronounced effects on steroid-induced mRNA expression at higher micromolar concentrations (as found in cervical tissue when used as a vaginal microbicide), in which case it might alter gene expression locally in the FGT.

4.4 *DPV and TDF have cell-specific effects on cell viability*

In contrast to their effects in U2OS cells (after 24 hours), in PBMCs, DPV and TDF reduced cell viability by approximately 5% and 6% relative to vehicle, respectively (after 48 hours) (**Fig. 3.6f**). Taken together, the suggestion of these results is that the ARVs alter cell viability in a cell-type and/or time-dependent manner, but the interpretation of these results is confounded by the fact that incubations in experiments in the two model systems were of different lengths. Given that the concentrations of DPV and TDF tested in the current study were up to 1000-fold higher than reported peak serum concentrations in individuals using these ARVs prophylactically (**Table 1.2**), the results of the current study suggest the ARVs are unlikely to alter cell viability systemically when used as PrEP. The concentrations of TDF tested approached peak serum concentrations in some individuals using the ARV therapeutically (see **Table 1.2**). In these individuals, the results of the current study suggest TDF may alter the viability of some systemic immune cells by a small degree, but whether this would alter their function, and if TDFs effects would be compounded over time is unclear. DEX reduced cell viability by approximately 20% in PBMCS, and there was no significant change in viability in the presence of the ARVs. The effects of DEX in these

experiments is consistent with other studies; DEX has long been known to induce apoptosis in lymphocytes (Migliorati *et al.*, 1994; Andreau *et al.*, 1998). It is unclear if reduced cell viability in PBMCs in the presence of the ARVs generally altered mRNA expression, but comparison of GAPDH mRNA levels in ARV- and non-ARV-treated PBMCs in these experiments (data not shown) suggests this was not the case. The inclusion of a cytotoxic positive control experiments assessing cell viability in the present study would increase the credibility of the observed results.

Findings in this study that DPV and TDF have cell-specific and/or time dependent effects on cell-viability are consistent with those previously reported by others, using different cell lines. The 50% cytotoxic concentration (concentration of the drug at which the cell viability was reduced to 50% of the drug-free control value) of DPV in TZM-bl cells, PM-1 CD4+ T cells and macrophages is approximately 10–20 μM after 14 days (Fletcher *et al.*, 2009). In cervical explants, and in the HEC-1A human endometrial adenocarcinoma cell line, TFV has been shown to have no cytotoxic effects at concentrations up to 100 μM after 24 hours (Gali *et al.*, 2010). Conversely, Grigsby *et al.* showed treatment of murine primary osteoblasts with TDF, at concentrations of 50 μM or more, over a period of 3 days significantly reduced their viability (Grigsby *et al.*, 2010). The present study did not evaluate the effects of higher micromolar concentrations of DPV and TDF on cell viability, as found in cervical tissue following intravaginal delivery of these ARVs, but from the findings of others, one can conclude the ARVs have dose-, time- and concentration-dependent effects on cell viability. The GCs, progestins and ARVs tested do not appear to modulate each other's effects on cell viability.

DEX, CORT and MPA have previously been shown to have differential, cell-line-dependent cytotoxic effects in various carcinoma cell lines (Sutherland *et al.*, 1988; Dran *et al.*, 1995; Lu *et al.*, 2005). The highest concentration of CORT used in this study (1 μM), induced an apparent decrease in cell viability of approximately 15%. Interestingly, in the presence of TDF, this apparent decline in viability was abrogated. Unlike other NRTIs, TFV has been shown by others to induce minimal or no mitochondrial toxicity in human hepatoblastoma (HepG2) cells, skeletal muscle cells, or renal proximal tubule epithelial cells *in vitro*, even following long-term incubations with supra-physiological concentrations (Birkus *et al.*, 2002; Venhoff *et al.*, 2007). Given these reports, and the lack of effect on cell viability observed in U2OS cells in this study, effects on mitochondrial function are unlikely to be a mechanism through which the relatively low concentration of TDF affects steroid function in this *in vitro* study. The broader implications of ARV effects on cell viability in the presence of steroid in the FGT and systemically are difficult to extrapolate, given

GCs, progestins and ARVs have highly varied, cell-, dose- and time-dependent effects on cell viability.

4.5 *Effects of MVC, DPV and TDF on GR-mediated mRNA expression in PBMCs*

This study investigated the capacity of MVC, DPV and TDF to alter GR-regulated immunoregulatory gene mRNA expression in PBMCs (**Fig. 3.6**). In PBMCs from the majority (67%) of donors, the ARVs decreased basal GILZ mRNA expression after 48 hours (**Fig. 3.6a**). To the best of the present authors knowledge, this is the first study showing MVC, TDF and DPV reduce basal GILZ mRNA expression in PBMCs. These findings were in contrast to observations in U2OS cells; although TDF decreased DEX-induced GILZ mRNA levels, neither DPV nor TDF reduced basal GILZ mRNA expression (after 24 hours) (**Fig. 3.4a**). Therefore, it would appear the ARVs have cell- and/or time-dependent effects on GILZ mRNA expression, but this cannot be determined conclusively from the results of this study, because incubations in experiments in the two model systems were of different lengths. The ARVs did not significantly alter DEX-induced GILZ expression in PBMCs from the majority of donors. Differing responses in PBMCs and U2OS cells could be explained by differential co-regulator, transcription factor and/or signaling protein expression, differences in responses to endogenous and exogenously expressed GR, or varying GR levels. GILZ is a critical mediator of the anti-inflammatory and immunosuppressive effects of GCs, through preventing the transcription of pro-inflammatory cytokines and preventing activation in macrophages (Berrebi *et al.*, 2003; Riccardi, 2010). Hladik *et al.* showed a 1% TFV gel reduced (GR-transactivated) IL-10 mRNA expression in rectal biopsies, which was associated with TFV-induced downregulation of CREB and CBP (Hladik *et al.*, 2015). It is possible TDF has similar effects on CREB and CBP expression in PBMCs, which would explain how it reduces basal GILZ mRNA expression in these cells.

Consistent with decreasing basal GILZ mRNA levels, when examining pooled data, the ARVs appeared to increase basal IL-6 and IL-8 mRNA expression in PBMCs. Therefore, in PBMCs, the ARVs appear to alter GR-regulated mRNA expression in a gene-dependent manner. The effects of the ARVs on cytokine and chemokine gene expression observed in PBMCs in the present study are consistent with their effects in other cell types, reported by others. TFV has been shown to increase IL-8 expression in endometrial and endocervical cells, and DPV has been reported to induce small increases in IL-8 in some FGT cell lines, genital ECs and cervical tissue *in vitro* (Gali *et al.*, 2010; das Neves *et al.*, 2013 Biswas *et al.*, 2014). TDF selectively modulates cytokine

production in human PBMCs, consistent with the results of the present study; after TLR stimulation or stimulation with TNF-α, TFV has been shown to decrease IL-10, and increase pro-inflammatory IL-12 gene expression *in vitro,* suggesting TFV and immune activators may co-regulate cytokines in a synergistic manner *(*Melchjorsen *et al.*, 2011).

In this study, MVC had marked pro-inflammatory effects in PBMCs from 44% donors; simultaneously decreasing basal GILZ mRNA expression and increasing basal IL-6 and IL-8 mRNA expression by 2-fold or greater (see Appendix **Fig. A7-A9**). DPV and TDF had similar effects in PBMCs from 10% and 20% of donors, respectively. Interestingly, the observation that MVC had proinflammatory effects in the more donors than did DPV or TDF is consistent with reports that MVC activates NF-κB via binding to CCR5, consequently inducing proinflammatory cytokine expression in CD4+ T cells (Madrid-Elena *et al.*, 2018). In combination with DEX, the ARVs proinflammatory effects were mostly abolished, which is consistent with the GC-bound GR antagonizing the activity of AP-1, NFAT and NF-κB (De Bosscher *et al.* 2000; Nissen and Yamamoto, 2000). Like DPV and TDF, MVC was found to not transcriptionally activate the GR in an *in vitro* COS-1 cell line model, alone or in the presence of DEX, MPA or LNG in the present study (Appendix **Fig. A2**). COS-1 cells do not express CCR5 (Geiger *et al.*, 2012), so in these cells, MVC most likely has no effects on GR-regulated gene expression caused by activating NF-κB. In addition to potential mechanisms outlined elsewhere in this chapter, a mechanism by which DPV and TDF may induce their pro-inflammatory effects is through modulating NF-κB activity, as has been reported with MVC. However, Melchjorsen *et al.* found no effect of TDF on NF-κB activity in monocytes (Melchjorsen *et al.*, 2011). In PBMCs from all the donors in which the combined pro-inflammatory effects on GILZ, IL-6 and IL-8 mRNA expression outlined above were observed, the ARVs also increased basal IFN-γ expression. IFN-γ has varied immunoregulatory effects, including inducing lymphocyte activation, antigen presentation and pro-inflammatory antiviral responses (Roff *et al.*, 2013). Although its ability to inhibit HIV-1 replication in monocytes and macrophages (Kornbluth *et al.*, 1990; Roff *et al.* 2013) may be protective against HIV-1 infection *in vivo,* it's enhancement of lymphocyte activation and recruitment may increase the risk of HIV-1 acquisition (Roff *et al.* 2013).

Chronic immune activation and inflammation drive HIV-1 disease progression and are also associated with an increased risk of cardiovascular and liver disease, cancer and metabolic disorders, and therefore non-AIDS mortality (Currier *et al.*, 2008; Deeks, 2011; Manabe, 2011; Smith *et al.*, 2014; Korniluk *et al.*, 2017). Systemically, and in the FGT, inflammation and immune

activation are associated with an increased risk of HIV-1 acquisition (Fowke *et al.*, 2012; Card *et al.*, 2013; Masson *et al.*, 2015; McKinnon *et al.*, 2018). Therefore, if the effects observed in the presence of ARVs in PBMCs in this study are translated *in vivo*, and assuming they persist indefinitely, the implication is that DPV, TDF and MVC may have proinflammatory effects in some individuals, which may undermine their effectiveness when used prophylactically, through enhancing HIV-1 target cell recruitment and activation, and inducing inflammatory tissue damage. Theoretically, the ARVs anti-HIV-1 activity mitigates the increased susceptibility to HIV-1 infection posed by their proinflammatory effects, but not susceptibility to the acquisition of other STIs. Conversely, enhanced immune activation and immune cell recruitment may lead to augmented responses to pathogens, thereby reducing the risk of HIV-1 acquisition. Confounding interpretation of these results is the fact that IL-8 and IFN-γ have been reported to both stimulate and inhibit HIV-1 infection (Kornbluth *et al.*, 1990; Mackewicz *et al.*, 1994; Lane *et al.*, 2001; Roff *et al.* 2013), and that IL-6 may have both proinflammatory and anti-inflammatory effects, which are dependent on the levels and identity of other cytokines present (Xing *et al.*, 1998; Tanaka *et al.*, 2014).

Proinflammatory effects were abrogated in the presence of DEX, which supports suggestions by others that the inclusion of immunosuppresive agents in microbicides and PrEP may mitigate the increased susceptibility to HIV-1 infection posed by their proinflammatory effects (Naranbhai *et al.*, 2012). In some individuals using TDF and MVC therapeutically, this study suggests these ARVs may contribute to the chronic inflammation and immune activation which are central to the pathogenesis of HIV-1 infection or may increase the risk of non-AIDS mortality. It should be noted that the downstream effects of the present studies *in vitro* findings are difficult to determine and would require further study of resulting downstream signaling and cytokine networks to determine *in vivo* effects.

PBMCs abundantly express the GR endogenously (Cabrera-Munoz *et al.*, 2012; Tomasicchio *et al.*, 2013), and some studies report detectable PR protein (Cabrera-Munoz *et al.*, 2012) while others do not (Tomasicchio *et al.*, 2013). Detectable AR and MR protein have not been reported in PBMCs (Cabrera-Munoz *et al.*, 2012; Tomasicchio *et al.*, 2013). It is possible the presence of PR in PBMCs may influence the activity of the ARVs, but this has not been investigated. ARV effects on IL-6, IL-8, and IFN-γ mRNA expression were variable and donor-specific, which is not surprising; it is well established that PBMC responses are highly variable and dependent on multiple physiological factors, including the presence of infection, inflammation, age, race,

nutritional status, concentrations of circulating hormones and the relative proportion of specific cell populations (Longo *et al.*, 2012; Klein and Flanagan, 2016). In addition, varying GR/PR levels between donors may have contributed to the variable, donor-specific effects observed in this study, but this was not investigated. Although all PBMC donors were negative for HIV-1, syphilis and hepatitis A and B, they may have had other infections which were not tested for, which may have influenced their immune activation status and therefore immunoregulatory gene expression. With the exception of age and sex of PBMC donors, other details regarding donors were not available; it was therefore not possible to make any population-level inferences from the data.

. 4.6 *Steroid effects on the antiviral efficacy of DPV and TDF in PBMCs*

Encouragingly, this study found that neither DEX nor MPA significantly affected the antiviral efficacy of DPV or TDF in PBMCs from all three donors tested, at concentrations higher than those typically reported in the serum of users of these steroids (see **Table 1.1 & 1.3**). LNG did not affect the inhibitory efficacy of DPV in PBMCs from all three donors tested. However, LNG decreased the inhibitory efficacy of TDF in PBMCs from one donor. The results of this study suggest that at physiological concentrations, in the majority of individuals, GCs and progestins are unlikely to affect the anti-HIV-1 efficacy of PrEP ARVs *in vivo*. When steroids do affect the anti-HIV-1 efficacy of ARVs, the effect appears to be individual- and steroid-specific, but interpretation of the results of these experiments is limited by the relatively low number of donors for which effects were assessed. The implications of these findings in HIV-1 positive women using HAART, which involves the use of different ARVs in combination, are unclear. Moreover, ARVs may be present together with multiple steroids, for example MPA and CORT in DMPA users, combinations of which were not assessed in this study.

Contrary to the results of the present study, Shen *et al.* found MPA, but not LNG or P_4, suppressed the anti-HIV effect of TFV by reducing intracellular TFV-DP in peripheral blood CD4+ T cells (Shen *et al.*, 2017). In genital CD4+ T cells, this group found MPA decreased TAF (a prodrug of TFV) inhibition of HIV-1 infection and lowered TFV-DP concentrations, suggesting MPA influences the efficacy and potency of ARVs in an ARV- and cell-specific manner (Shen *et al.*, 2017). These findings, and those of the current study, suggest progestogens modulate the antiviral effects of TDF in a steroid-specific manner. However, whether the *in vitro* changes described above correlate to decreased HIV-1 protection *in vivo* remains to be determined. Of concern, in this study, the presence of LNG appeared to decrease the antiviral efficacy of TDF in PBMCs from

one donor. In contrast, Shen *et al.* showed no effect of LNG on intracellular TFV-DP levels or TFV antiviral activity in CD4+ T cells, as observed in PBMCs from two of three donors in the present study (Shen *et al.*, 2017). PBMCs are comprised of a heterogenous population of HIV-1 target cells, including monocytes and macrophages, as well as CD4+T cells (Autissier *et al.*, 2010). Macrophages, like CD4+ T cells are productively infected by HIV-1 and contribute to the infection of CD4+ T cells (Waki and Freed, 2010). There are no studies which have determined the effects of progestins on TFV-DP in monocytes or macrophages specifically, which may account for the variance between the present study and published literature.

There are no published studies assessing the effects of DEX or CORT on ARV antiviral efficacy. However, a study has shown pretreatment of macrophages with E_2 and P_4 induced dose-dependent decreases in proinflammatory and antiviral responses, correlated with reduced HIV-1 replication in the absence of ARVs, suggesting the immunosuppressive effects of steroids via steroid receptors may complement the antiviral effects of ARV (Devadas *et al.*, 2018). Some progestin-based HCs are administered in combination with estrogenic compounds (Sitruk-Ware, 2006; Sitruk-Ware and Nath, 2010). There is evidence suggesting E_2 may be protective against HIV-1 infection. For example, E_2 treatment has been shown to be protective against vaginal SIV transmission in macaques (Smith *et al.*, 2000) and is protective against HIV-1 infection in human CD4+ T cells and macrophages *in vitro* (Rodriguez-Garcia *et al.*, 2013). It is possible E_2 in HCs is similarly protective against HIV-1 infection in women, although this remains to be determined. If so, the inclusion of E_2 in MPTs may mitigate the inflammatory effects of the ARVs and any increased risk this may pose to HIV-1 acquisition.

4.7 *Limitations of the study*

This study has several limitations, which may limit the applicability of the observed results to those *in vivo*. *In vivo* testing in humans undoubtedly allows determination of physiologically relevant outcomes, but one cannot directly determine intracellular mechanisms of action. *In vitro* experiments, however, have the distinct advantage of allowing assessment of cellular intrinsic responses and the intracellular mechanisms involved. Thus, this thesis used *in vitro* models to investigate proof-of-concept hypotheses and mechanisms and interpreted the results with appropriate caution.

Immortalized cells, like the U2OS cell line used in this study, unlike primary cells, avert senescence through bypassing cell cycle checkpoints, often through the expression of oncogenes (Maqsood *et al.*, 2013). As a result, some cell signaling and transcriptional responses in immortalized cells can differ from those in primary cells (reviewed by Kaur and Dufour, 2012). However, not all responses differ; for instance, DEX and MPA have been shown to have similar effects on GR-regulated genes, including GILZ, IL-6 and IL-8, in immortalized endocervical cell lines, cervical explants and PBMCs (Govender *et al.*, 2014; Ray *et al.*, 2014). Moreover, many mechanisms have been identified in immortalized cells and have been shown to be consistent with *in vivo* results, such as the anti-inflammatory receptor-mediated cellular mechanisms of GCs (Fauci and Dale, 1974; Guichard *et al.*, 2015), the intracellular mechanism of action of ARVs (Fletcher *et al.*, 2009; Rohan *et al.*, 2010), and steroid-receptor activity and gene expression effects of progestogens currently in used in patients (Goldfien *et al.*, 2015). The U2OS cell line has a large number of structural and numerical chromosomal alterations (Bayani *et al.*, 2003), and expresses several proto-oncogenes, which may influence some transcriptional and cell signaling responses (Niforou *et al.*, 2008). Whether GR-mediated responses determined *in vitro* in U2OS cells in this study occur in all cells expressing the GR *in vivo* remains to be determined. Nevertheless, many GC-mediated responses in U2OS cells are similar to those in primary cells, so it was a suitable model for use in the current study. For example, GCs and progestins have been shown to induce similar changes in GILZ mRNA expression in PBMCs and U2OS cells (Hadley *et al.*, 2011; Govender *et al.*, 2014; Ray *et al.*, 2014). and the mechanisms through which some cellular stressors desensitize GC signaling via the GR were first identified in U2OS cells (Galliher-Beckley *et al.*, 2011).

Limitations of promoter-reporter assays, notably the absence of chromatin and the ratio of promoter-reporter constructs to transcription factors, have been discussed elsewhere in this chapter. Nevertheless, many results obtained with reporter assays have helped to identify transcriptional mechanisms that also occur on endogenous genes. For example, transcription elements regulating the expression of IL-2, IL-5 and CCR5 by the GR were identified using reporter genes (Bamberger *et al.*, 1997; Zhang *et al.*, 1997; Guignard *et al.*, 1998). Transcriptional activity on reporter genes is often similar to that on endogenous genes; for example, Ronacher *et al.* showed similar effects of GCs and progestins on GILZ mRNA expression and a GRE-reporter gene in COS-1 and U2OS cells (Ronacher *et al.*, 2009). A useful strategy, as employed in this thesis, is to compare results on reporter assays with those on endogenous genes. Determining GR-regulated mRNA levels, although more physiologically relevant than reporter assays, carries its own limitations, so caution must be taken when extrapolating effects on mRNA expression to

phenotypic responses. This study determined changes in immunomodulatory gene mRNA expression, which may not necessarily correlate with transcriptional effects or changes in protein levels due to multiple mRNA post transcriptional mechanisms (Liu *et al.*, 2016). Although mRNA post-transcriptional regulation is a critical determinant of final protein concentrations, particularly under conditions like differentiation or stress responses, under steady state conditions, mRNA abundance does primarily determine protein abundance (Liu *et al.*, 2016).

Effects induced by ARVs in U2OS cells and PBMCs in the present study have in some instances been extrapolated to *in vivo* effects on HIV-1 susceptibility and HIV-1 disease progression. It should be acknowledged that there are other more physiologically relevant models which would have been better suited to making such *in vitro*-to-*in vivo* extrapolations. For example FGT cell lines expressing the GR endogenously like the End1/E6E7 and HeLa FGT cell lines , or cervical explants, would have been better *in vitro* models from which effects on HIV-1 susceptibility in the FGT *in vivo* could be extrapolated. However, these models have some disadvantages when attempting to establish proof-of-concept hypotheses and mechanisms via steroid receptors, as in the present study. Unlike steroid deficient U2OS cells, most cells express multiple steroid receptors, which would confound interpretation of the results of this study. For example HeLa and like End1/E6E7 cells have been shown to express the GR and MR endogenously (Govender *et al.*, 2014), and these two steroid receptors are known compete for the same ligands, bind to the same response elements, transcription factors and co-regulators, and can heterodimerize (Gomez-Sanchez and Gomez-Sanchez, 2014). In addition to expressing all steroid receptors (Ray *et al.*, 2019), cervical explants are far less accessible than U2OS cells or PBMCs. Another limitation of the current study is that it is not clear how the amount of GR transfected into U2OS cells compares to that in cells expressing the GR *in vivo*.

Whether responses observed in PBMCs *in vitro* in this study are replicated in immune cells *in vivo* remains to be established. It should be noted that responses in cultured PBMCs may differ from those *in vivo*, as the activity and activation status of PBMCs *in vivo* is influenced by numerous dynamically expressed cytokines and chemokines, as well as the endocrine system (Watkins *et al.*, 1999). It should also be considered that the phenotype and responses of immune cells found in lymphoid organs and the mucosal surfaces of the FGT and gut (which are most relevant to HIV-1 transmission and disease progression), are distinct from immune cells in peripheral blood (Brenchley *et al.*, 2004; Trifonova *et al.*, 2014). Therefore, care must be taken when drawing conclusions from effects observed in PBMCs regarding HIV-1 susceptibility or disease

progression. Nevertheless, PBMCs consist of many of the lymphocyte, DC and macrophage subsets relevant to HIV-1 transmission and infection (Autissier *et al.*, 2010). Indeed, much of what is known regarding CD4+ T cell depletion, T cell activation, T cell dynamics, and HIV-specific immune responses in HIV infection was first elucidated from the analysis of PBMCs (Clark *et al.*, 1991; Koot *et al.*, 1992; Roederer *et al.*, 1995; Haase, 1999).

Another limitation of the present study is that infection assays made use of HIV-1 infectious molecular clones, whereas HIV-1 transmission in women typically occurs a following exposure to infected semen; a complex mixture of cells and immunoregulatory molecules, which actively modulates inflammation, immune cell activation and the efficiency of HIV-1 transmission in the FGT (Sharkey *et al.*, 2012; Introini *et al.*, 2017). The use of only infectible PBMCs (determined to be infectible by prescreening for infectability) may have biased the results of infection assays by selecting for donors with immune profiles at higher risk of HIV-1 infection. However, the reasons for non-infectability of isolated PBMCs not selected may not have been due to intrinsic donor factors, but due to subsequent damage during PBMC storage and isolation. Furthermore, this study did not assess the immunomodulatory effects of the ARVs in HIV-1-infected PBMCs, which would be more relevant to determining any effects on HIV-1 disease progression, and may differ from those in uninfected PBMCs due to their heightened state of activation (Ford, Puronen and Sereti, 2009). It is possible steroids differentially alter the ARVs anti-HIV-1 effects against X4 tropic virus, which was not investigated in this study. Given that HIV-1 has been shown to convert from R5 tropic to X4 tropic with time in approximately 50% of HIV-1 infected individuals, determining the effects of progestins and GC on the efficacy of ARVs against X4 tropic viruses is important, but remains unexplored (Berger *et al.*, 1999; Regoes and Bonhoeffer, 2005; Mosier, 2009). This study did not investigate the effects of DPV, TDF or MVC on GR-regulated mRNA expression in PBMCs in the presence of CORT, MPA or LNG, which may differ from effects in the presence of DEX (as was observed in U2OS cells). This would have required PBMCs from more donors than were available for this study and was beyond the scope of this study. As such, from the results presented in this thesis, one cannot conclusively state the effects of the ARVs studied on GC- and progestin-induced immunomodulatory gene expression. Furthermore, in most experiments, the effects of a single ARV concentration, at a single time-point, were investigated; testing ARV effects at a range of concentrations spanning those reported *in vivo* (see **Table 1.2**), and at different time points, may be more informative regarding their possible *in vivo* effects on HIV-1 susceptibility and HIV-1 disease progression.

CHAPTER 5
CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 *Conclusions*

This study has shown that DPV and TDF do not independently transcriptionally activate the GR, but increase GC transcriptional efficacy via the GR on a reporter gene *in vitro*, which is a novel finding. These findings, if extrapolated *in vivo*, suggest DPV and TDF may potentiate the transactivational effects of GCs via the GR during times of stress, or GC therapy, resulting in adverse side effects, for example osteoporosis (Baschant and Tuckermann, 2010; Ramamoorthy and Cidlowski, 2013). The results of the current study suggest TDF, but not DPV, may modulate DEX-induced anti-inflammatory gene expression during GC therapy or CORT- and MPA-induced anti-inflammatory gene expression under cellular contexts where they act as a full agonists for the GR (Ronacher *et al.*, 2009). As such, the use of TDF prophylactically or therapeutically may result in a decreased ability to blunt immune responses and therefore increase inflammation and immune activation. This may increase the risk of HIV-1 acquisition or promote HIV-1 disease progression and non-AIDS mortality (Deeks, 2011). In the present study, DPV and TDF did not significantly alter GR protein levels, or cell viability, alone or in the presence of steroid, in the cell line model, suggesting alterations in GR concentrations or cell viability are not likely to be the mechanisms through which the ARVs alter GR-mediated activity. This study showed TDF induced apparent increases in DEX-induced GR phosphorylation at Ser226, to a greater extent than did DPV. This is consistent with findings in this study that TDF, but not DPV, decreased DEX-induced anti-inflammatory gene mRNA expression in U2OS cells, as phosphorylation at this residue is associated with blunted GR transcriptional responses (Kino *et al.*, 2007).

In PBMCs from some donors, MVC, DPV and TDF had marked proinflammatory effects; simultaneously increasing pro-inflammatory cytokine mRNA expression and decreasing anti-inflammatory GILZ mRNA expression. If similar effects occur in the FGT, in some women, PrEP ARVs may paradoxically increase the risk of acquiring sexually transmitted infections, including HIV-1, through increased recruitment and activation of HIV-1 target cells, and enhancing inflammation. Conversely, these women may benefit from enhanced immune responses against pathogens. In individuals using MVC and TDF therapeutically, the proinflammatory effects of the ARVs may contribute to HIV-1 disease progression, as well as non-AIDS mortality (Deeks, 2011).

In the presence of DEX, most ARV-induced proinflammatory effects were abrogated, suggesting the inclusion of an immunosuppressive agent in microbicides and MPTs may mitigate the unwanted proinflammatory effects of PrEP ARVs.

This study found that DEX, MPA and LNG did not alter the antiviral efficacy of DPV or TDF in PBMCs from the vast majority of donors tested. Taking the results of the current study and those of others into consideration, DPV is proposed to be more suitable ARV for MPTs and microbicides than TDF or MVC. TDF was shown in this study to modulate GC-induced mRNA expression, whereas DPV did not. Furthermore, DPV induced pro-inflammatory changes in mRNA expression in PBMCs from less donors than did the other ARVs, which further favors its use for PrEP. Moreover, previous studies have found MPA reduces the antiviral efficacy of TDF in CD4+ T cells (Shen *et al.*, 2017), and the long-term use of TDF is associated with adverse effects on bone (Grigsby *et al.*, 2010; Komatsu *et al.*, 2018). No such adverse effects have been reported with the use of DPV (Chen *et al.*, 2015; Baeten *et al.*, 2016). The combination of LNG and DPV may be the best choice for MPTs, considering evidence from clinical and biological studies indicating that MPA use is associated with increased risk of HIV-1 acquisition (Hapgood *et al.*, 2018). In this study, MVC induced pro-inflammatory effects in PBMCs from more donors than did DPV or TDF. MVC has been shown to induce proinflammatory cytokine expression via NF-κB activation, and, as an early vaginal microbicide candidate, MVC was not protective against HIV-1 infection in cervical tissue (Chen *et al.*, 2015; Fletcher *et al.*, 2016; Madrid-Elena *et al.*, 2018). Therefore, of the three ARVs whose immunomodulatory effects were assessed in the current study, MVC appears to be least suitable for PrEP.

5.2 *Future perspectives*

Given the relatively small apparent differences and inherent technical and biological error in the experiments presented in the current study, it was not possible to establish whether the apparent differences observed were significant or not. It is therefore necessary that some experiments be repeated, or that more experiments with fewer variables be performed. The following questions remained unresolved in this thesis:

1. **Do the ARVs alter steroid efficacy or potency for transrepression via the GR?** To fully elucidate their actions via the GR, it is necessary to determine the ARVs effects on steroid-

induced transrepression via the GR. This could be achieved using NF-κB- and AP-1-reporter genes and assessing mRNA expression of GR-transrepressed genes.

2. **Are the effects of TDF on GC-induced mRNA expression gene-, steroid-, cell-, time-, and/or dose-dependent?** To provide clarity as to whether TDF has gene-specific effects, the effects of TDF on different GC-regulated genes, could be investigated. Ideally, one would assess the effects of TDF on the entire cell transcriptome, in the presence and absence of steroid, for example using microarray analysis. The current study suggested the effects of TDF on GC-induced gene expression *in vivo* may contribute to its adverse effects on BMD in some users (Grigsby *et al.*, 2010; Komatsu *et al.*, 2018). However, the effects of TDF on the expression of GC-induced genes which regulate BMD, for example RANKL and OPG (reviewed by Boyce and Xing, 2007), was not investigated; doing so would better inform this assumption. It is also important to determine if the changes in mRNA expression observed in this study are translated into changes in protein levels, using western blotting. TDF-induced repression of steroid-induced GILZ mRNA expression appeared to be dependent on the efficacy of the GR agonist in U2OS cells. It is important these experiments be repeated, possibly with fewer variables, to determine if the effects are steroid-specific. These experiments could be performed in different cell lines, including ones expressing the GR endogenously, for example HeLa or End1/E6E7 cells, to determine whether TDF alters GR-regulated gene expression cell-specifically. It would be interesting to determine the effects of the ARVs on CORT- and MPA-induced gene expression in cell lines where these steroids induce GR transactivation to a degree approaching that of DEX, for example COS-1 cells (Ronacher *et al.*, 2009). It would also be interesting to determine if GR protein levels in PBMCs from donors correlate with the donor-specific effects of the ARVs on immunomodulatory gene expression, and whether these ARV responses are cell-specific by cell-typing and quantification. The effects of DPV and TDF on GR-regulated gene expression, at several concentrations and time points, should be investigated to determine if their effects are dose- and/or time-dependent. It is possible pre-treatment of cells with ARVs prior to exposure to steroids would alter the results, given that on their own, the ARVs had immunomodulatory effects, which may impact steroid activity. This would require further investigation to determine.

3. **How do DPV and TDF alter cell viability in PBMCs?** In this study DPV and TDF significantly reduced cell viability in PBMCs. As the MTT assay (employed in experiments

assessing the effects of the ARVs on cell viability) measures metabolic activity of cells based on NAD(P)H-dependent enzymes, it may reflect changes in the metabolic state of the cell, cellular proliferation and/or cell death. Further evidence is required to validate the conclusions of the MTT assays in this study. For example, cell counts before and after treatment to determine cell proliferation and death, cell cycle phase analysis and proliferation assays, could be performed. It would be interesting to determine in which cell populations death occurs and assess the expression of apoptotic markers, which could be achieved using flow cytometry. To determine if the effects of the ARVs on cell viability altered global gene expression, it would also be informative to assess the expression of several reference genes in ARV- vs non-ARV-treated cells.

4. **What is the mechanism through which DPV and TDF alter GR-mediated mRNA expression in U2OS cells and PBMCs?** The results of this study suggest the mechanism in U2OS cells may involve differential ARV-induced alterations in DEX-induced phosphorylation at Ser226 on the GR. Assessing the ARVs effects on CDK5 and JNK, which phosphorylate the GR at Ser226 (Kino, 2018) may inform on the mechanism through which this occurs. To further elucidate the ARVs effects on GR activity, phosphorylation at other serine residues (see **Fig. 1.4**), should be assessed by western blotting. The ARVs effects on nuclear translocation (using cell fractionation techniques and western blotting), GR cofactor recruitment (using ChIP) and changes in GR-regulated gene expression should be investigated in parallel. TDF has been shown to downregulate the expression of CREB, CBP and MAPK, which are involved in activating the GR (Hladik *et al.*, 2015). It would be interesting to determine if TDF has similar effects in U2OS cells and PBMCs by comparing co-regulator recruitment in DEX- and non-DEX-treated cells, in the absence and presence of the ARVs using co-regulator microarray assays. TDF has also been shown to modulate expression of genes involved in the Wnt, TGF-β and Hedgehog signaling pathways in murine osteoblasts (Grigsby *et al.*, 2010). Assesing the effects of TDF on these genes in U2OS cells and PBMCs may clarify the mechanism behind its effects on GR-regulated mRNA expression in these cells.

This study found the ARVs tested induced pro-inflammatory effects in PBMCs from some donors tested. To determine if pro-inflammatory effects observed occur via NF-κB activation, as has been shown with MVC by others (Madrid-Elena *et al.*, 2018), ARV effects on NF-κB recruitment to promoters (using ChiP), NF-κB nuclear translocation and

phosphorylation could be assessed. The experiments conducted in PBMCs in the current study provide no evidence that the effects observed are GR-dependent. To clarify this, experiments could be repeated with the addition of a GR antagonist, for example RU486, or GR small-interfering RNA knockdown. Furthermore, these experiments only assessed mRNA expression, which may not correlate to the amount of secreted protein. Secreted cytokines could be quantified over a time-course using enzyme-linked immunosorbent assays, which would assist in determining more physiologically relevant outcomes.

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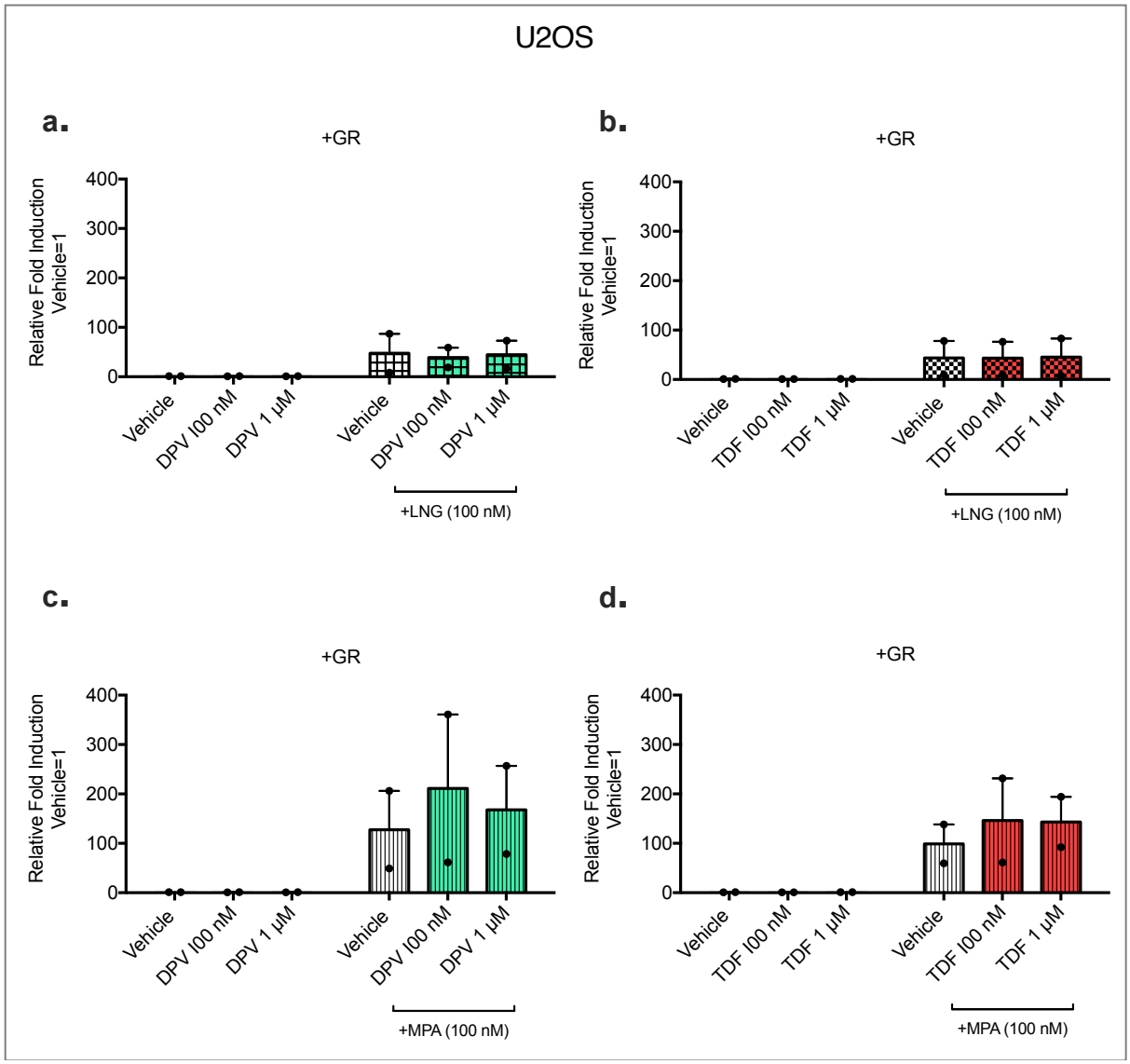

Figure A1: **DPV and TDF have no effect on the transcriptional activity of LNG or MPA via the GR.** U2OS cells were co-transfected with the GR expression vector (+GR) or its empty vector control (-GR), and the GRE luciferase reporter construct. Subsequently, cells were treated with DPV and TDF at the concentrations indicated in the presence or absence of 100 nM LNG (**a & b**) or MPA (**c & d**)**,** or vehicle (0.1% *v/v* EtOH and DMSO). Luciferase activity was normalized to protein content per well, as determined by a Bradford assay. Data are plotted as mean ± SEM, and are the pooled results of two independent experiments performed in triplicate.

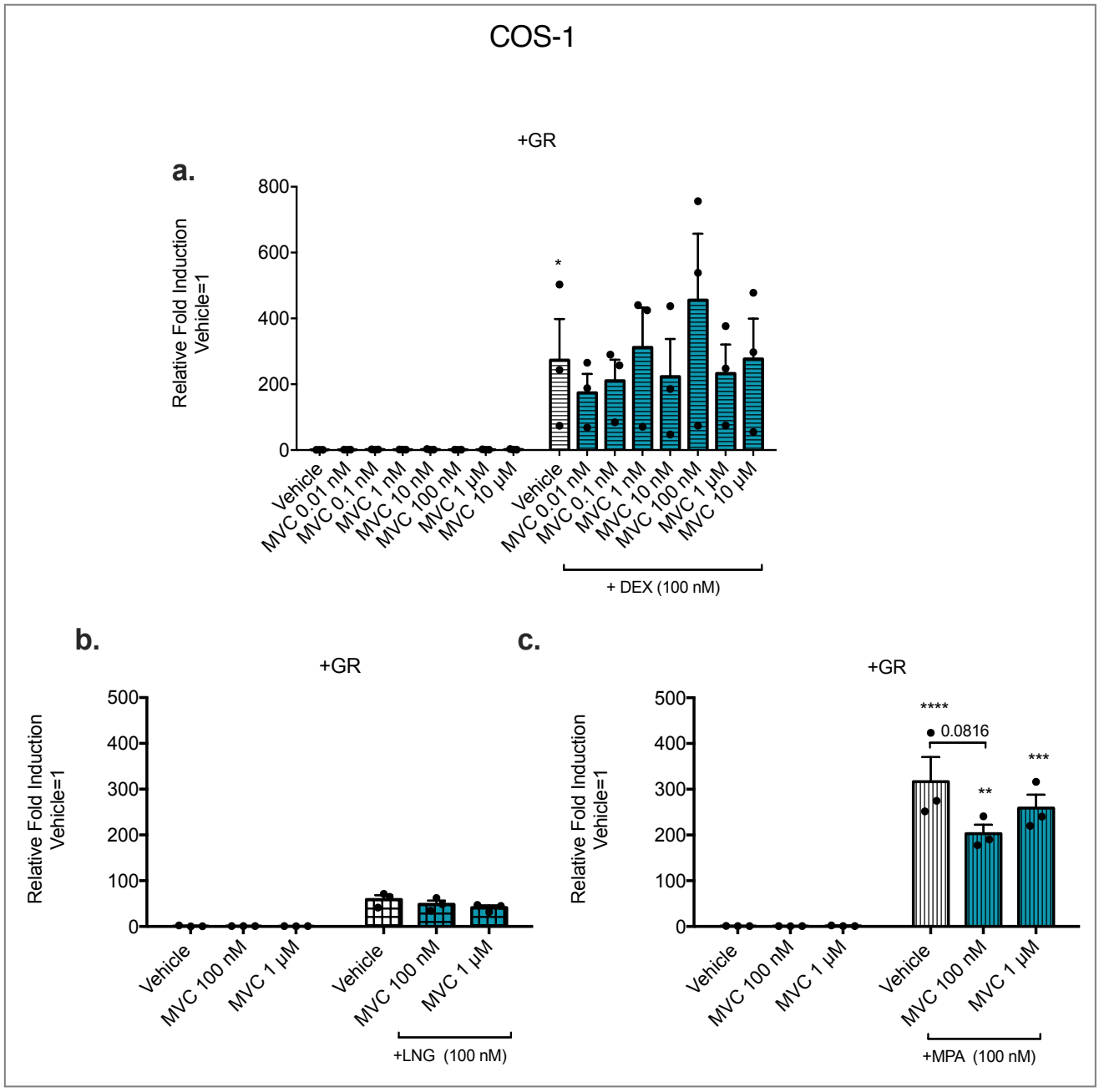

Fig A2: **MVC does not independently activate the GR, or alter the transcriptional activity of LNG or MPA via the GR.** COS-1 cells were co-transfected with the GR expression vector (+GR) or its empty vector control (-GR), and the GRE luciferase reporter construct. Subsequently, cells were treated with MVC at the concentrations indicated in the absence or presence of 100 nM DEX (**a**), LNG (**b**), MPA (**c**) or vehicle (0.1% *v/v* EtOH and DMSO) for 24 hours. Luciferase activity was normalized to protein content per well, as determined by a Bradford assay. Data are plotted as mean ± SEM, and are the pooled results of three independent experiments performed in triplicate. Statistical significance was determined using a two-way ANOVA with a Tukey's multiple comparisons post-test. Asterisks or p values above bars indicate significance compared to vehicle control (in the absence of steroid, black bar), unless otherwise indicated with lines. ****, ***, ** and * indicate $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

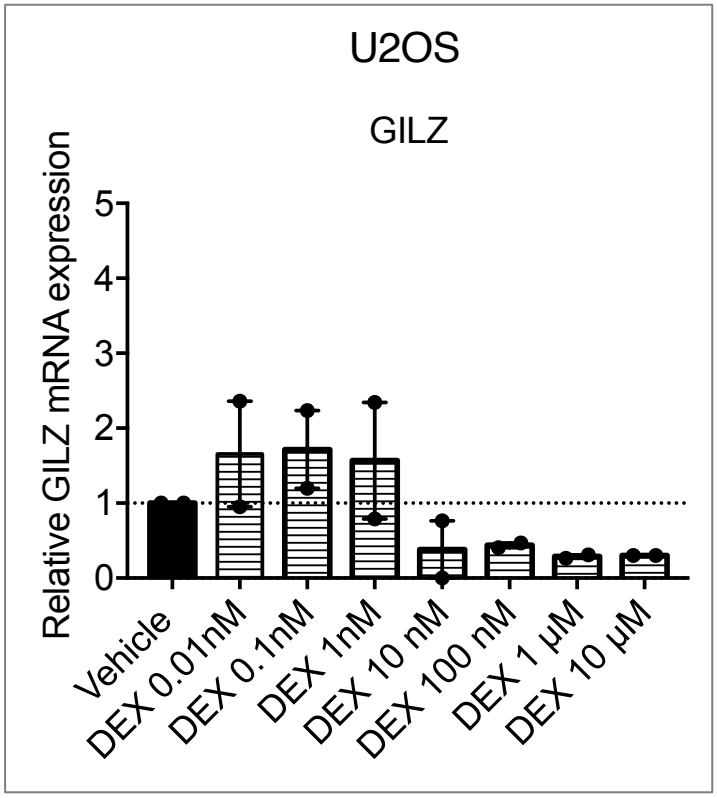

Figure A3: **DEX does not induce GILZ mRNA expression in U2OS cells, in the absence of the GR.** U2OS cells were transfected with pcDNA3.1; the empty vector control of the GR expression vector. Subsequently, cells were treated with increasing concentrations of DEX or vehicle (0.1% *v/v* EtOH and DMSO) for 24 hours as indicated. cDNA was synthesized and the expression of GILZ mRNA was determined, and normalized to GAPDH mRNA levels. Data are plotted as mean ± SEM and are the pooled results of two independent experiments. All experiments were performed in triplicate. Relative fold change in expression was determined by setting vehicle control to 1.

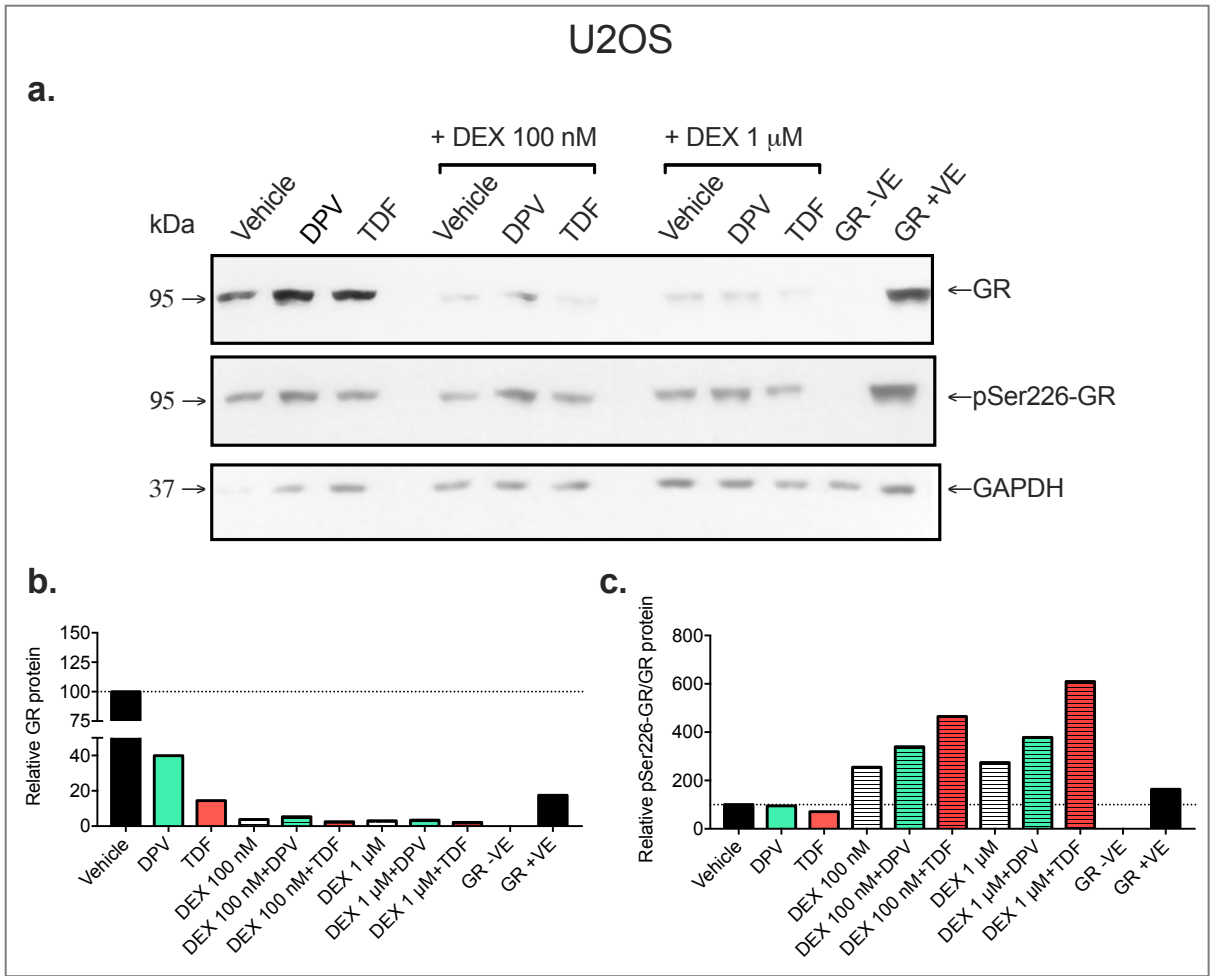

Figure A4. **DPV and TDF effect on GR protein levels and GR phosphorylation.** U2OS cells were transfected with the GR expression vector and subsequently stimulated with 1 μM DPV or TDF in the absence or presence of 100 nM DEX, 1 μM DEX or vehicle (0.1% *v*/*v* EtOH and DMSO) for 24 hours. Equal volumes of cell lysate were analysed by western blotting with GR and pSer226-GR specific antibodies, with GAPDH as a loading control. A representative western blot is shown in (**a**). Quantification of GR protein levels is shown in (**b**) and relative pSer226-GR in (**c**). Data are plotted as means.

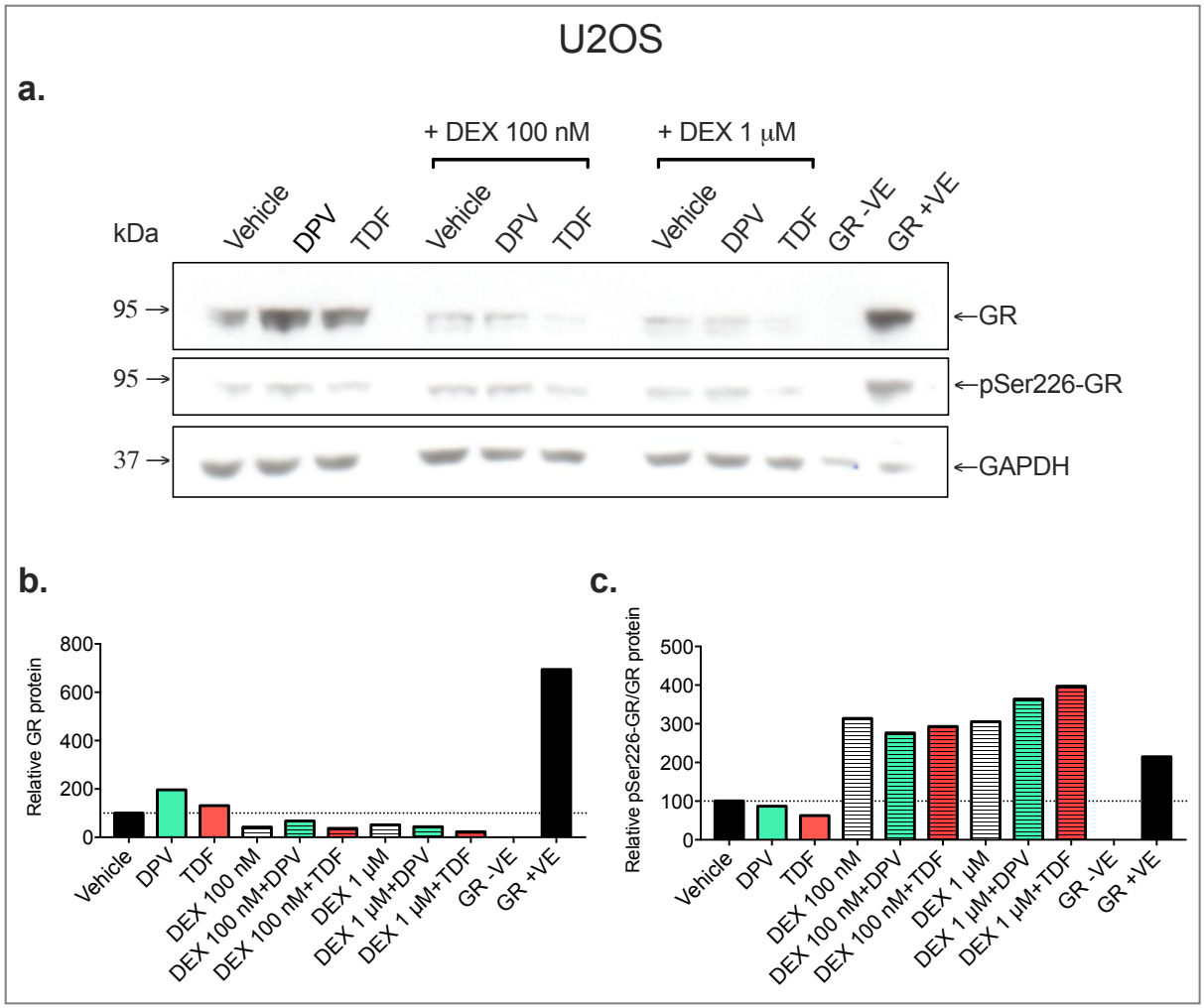

Figure A5. **DPV and TDF effect on GR protein levels and GR phosphorylation.** U2OS cells were transfected with the GR expression vector and subsequently stimulated with 1 μM DPV or TDF in the absence or presence of 100 nM DEX,1 μM DEX or vehicle (0.1% *v*/*v* EtOH and DMSO) for 24 hours. Equal volumes of cell lysate were analysed by western blotting with GR and pSer226-GR specific antibodies, with GAPDH as a loading control. A representative western blot is shown in (**a**). Quantification of GR protein levels is shown in (**b**) and relative pSer226-GR in (**c**). Data are plotted as means.

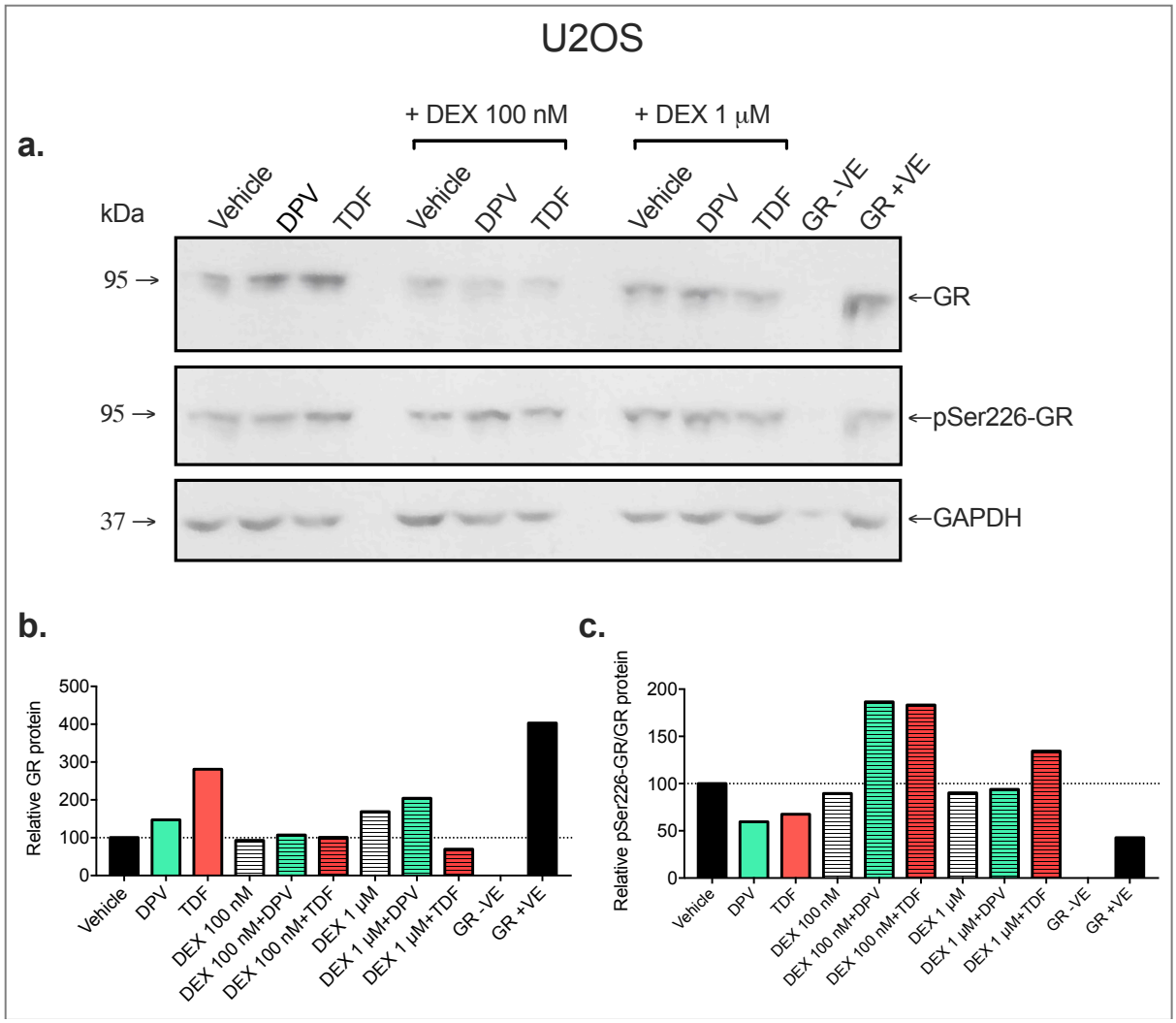

Figure A6. **DPV and TDF effect on GR protein levels and GR phosphorylation.** U2OS cells were transfected with the GR expression vector and subsequently stimulated with 1 μM DPV or TDF in the absence or presence of 100 nM DEX, 1 μM DEX or vehicle (0.1% *v*/*v* EtOH and DMSO) for 24 hours. Equal volumes of cell lysate were analysed by western blotting with GR and pSer226-GR specific antibodies, with GAPDH as a loading control. A representative western blot is shown in (**a**). Quantification of GR protein levels is shown in (**b**) and relative pSer226-GR in (**c**). Data are plotted as means.

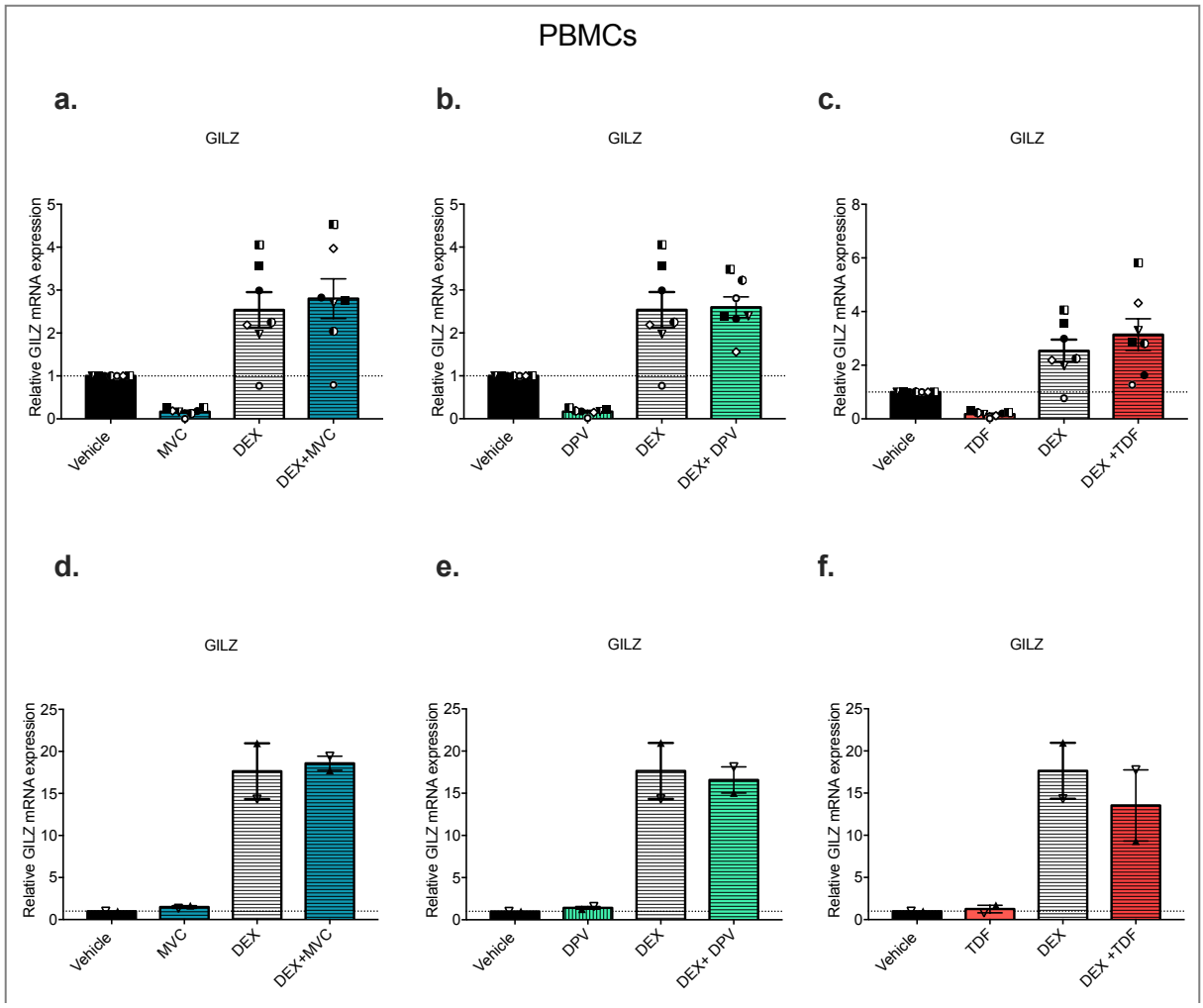

Figure A7: **DPV, TDF and MVC alter GILZ mRNA expression donor specifically.** PBMCs were stimulated with 1 μM MVC, DPV or TDF, in the absence or presence of 100 nM DEX, or vehicle (0.1% v/v EtOH and DMSO) for 48 hours, following which relative changes in GILZ mRNA expression were determined by real time qPCR, and normalized to GAPDH mRNA levels. Donors in which there was a 2 fold or greater decrease in GILZ mRNA expression upon treatment with MVC (**a**), DPV (**b**) and TDF (**c**) are shown in the top row, as are donors in which GILZ mRNA expression did not change by than 2-fold upon treatment with MVC (**d**), DPV (**e**) or TDF (**f**), in the bottom row. Relative fold change in expression was determined by setting vehicle control to 1. Data are plotted as mean ± SEM. Individual donors are depicted with specific black symbols.

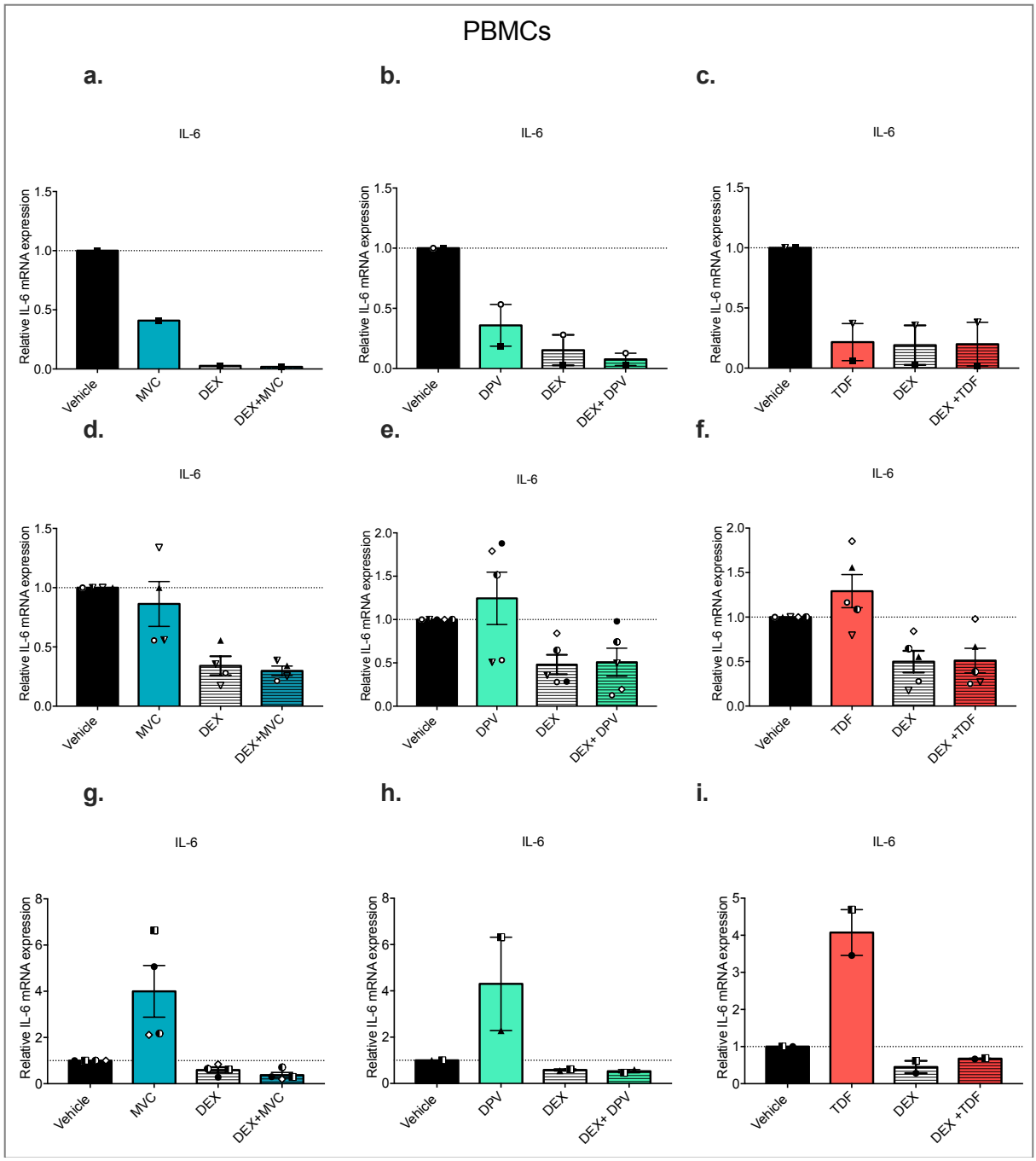

Figure A8: **DPV, TDF and MVC alter IL-6 mRNA expression donor specifically.** PBMCs were stimulated with 1 μM MVC, DPV or TDF, in the absence or presence of 100 nM DEX, or vehicle (0.1% *v*/*v* EtOH and DMSO) for 48 hours, following which relative changes in IL-6 mRNA expression were determined by real time qPCR, and normalized to GAPDH mRNA levels. Donors in which there was a 2 fold or greater decrease in IL-6 mRNA expression upon treatment with MVC (**a**), DPV (**b**) and TDF (**c**) are shown in the top row. Donors in which IL-6 mRNA expression changed less than 2 fold upon treatment with MVC (**d**), DPV (**e**) or TDF (**f**) are shown in the middle row. Donors in which there was a 2 fold or greater increase in IL-6 mRNA expression upon treatment with MVC (**g**). DPV (**h**) and TDF (**i**) are shown in the bottom row. Relative fold change in expression was determined by setting vehicle control to 1. Data are plotted as mean ± SEM. Individual donors are depicted with specific black symbols.

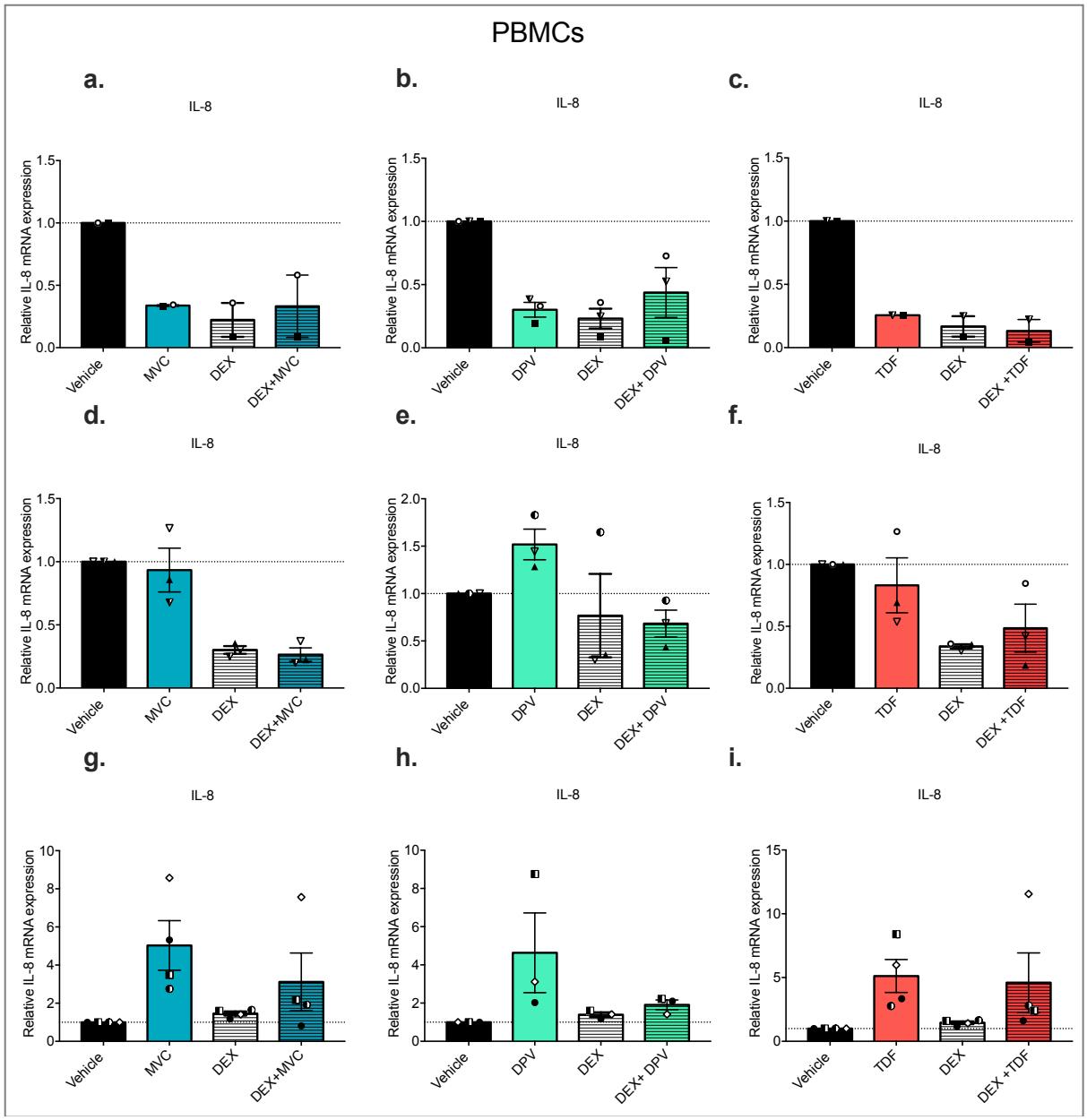

Figure A9: **DPV, TDF and MVC alter IL-8 mRNA expression donor specifically.** PBMCs were stimulated with 1 μM MVC, DPV or TDF, in the absence or presence of 100 nM DEX, or vehicle (0.1% *v/v* EtOH and DMSO) for 48 hours, following which relative changes in IL-8 mRNA expression were determined by real time qPCR, and normalized to GAPDH mRNA levels. Donors in which there was a 2-fold or greater decrease in IL-8 mRNA expression upon treatment with MVC (**a**), DPV (**b**) and TDF (**c**) are shown in the top row. Donors in which IL-8 mRNA expression changed less than 2-fold upon treatment with MVC (**d**), DPV (**e**) or TDF (**f**) are shown in the middle row. Donors in which there was a 2-fold or greater increase in IL-8 mRNA expression upon treatment with MVC (**g**). DPV (**h**) and TDF (**i**) are shown in the bottom row. Relative fold change in expression was determined by setting vehicle control to 1. Data are plotted as mean ± SEM. Individual donors are depicted with specific black symbols.

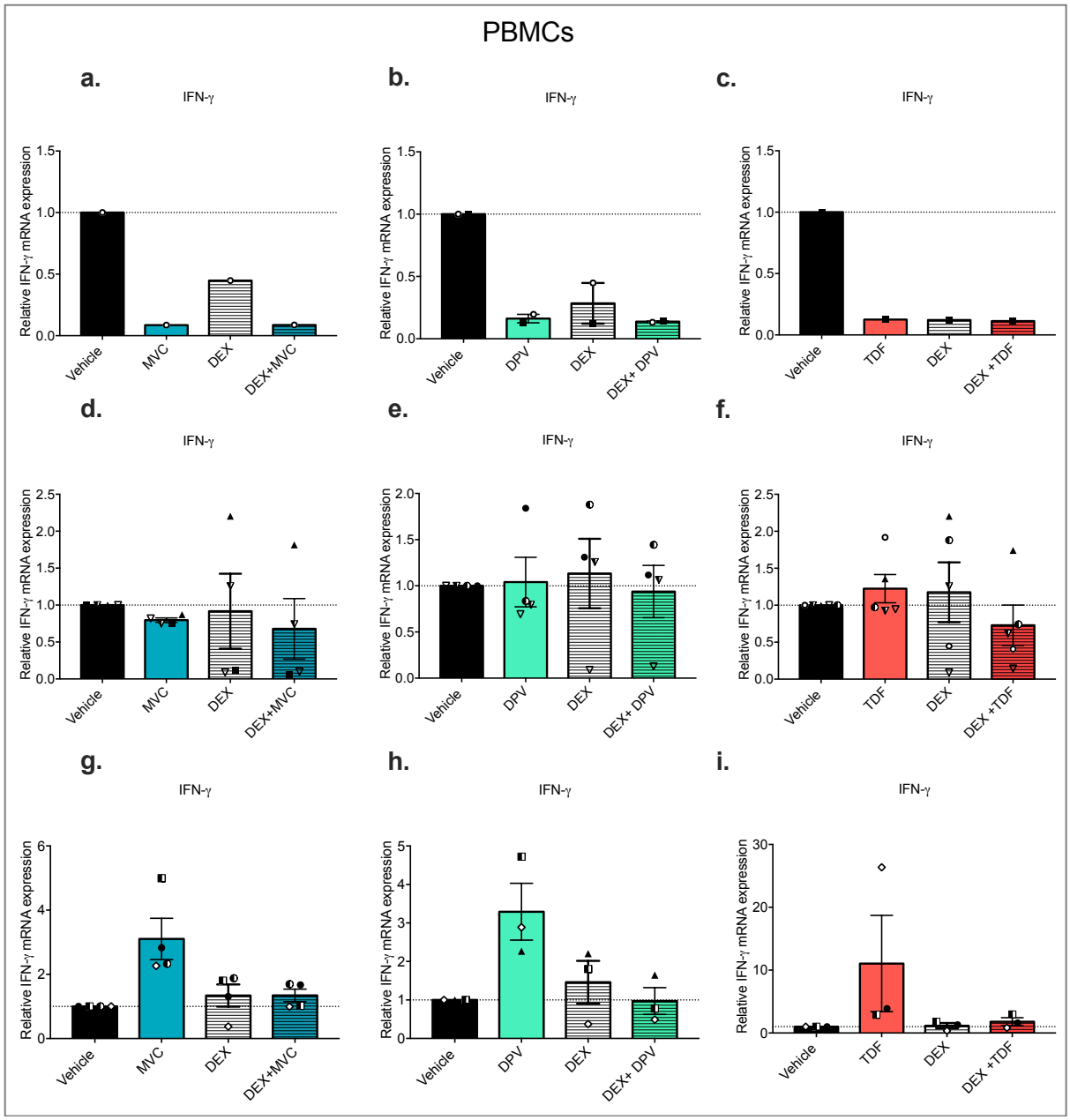

Figure A10: **DPV, TDF and MVC alter IFN-γ mRNA expression donor specifically.** PBMCs were stimulated with 1 μM MVC, DPV or TDF, in the absence or presence of 100 nM DEX, or vehicle (0.1% *v*/*v* EtOH and DMSO) for 48 hours, following which relative changes in IFN-γ mRNA expression were determined by real time qPCR, and normalized to GAPDH mRNA levels. Donors in which there was a 2-fold or greater decrease in IFN-γ mRNA expression upon treatment with MVC (**a**), DPV (**b**) and TDF (**c**) are shown in the top row. Donors in which IFN-γ mRNA expression changed less than 2-fold upon treatment with MVC (**d**), DPV (**e**) or TDF (**f**) are shown in the middle row. Donors in which there was a 2-fold or greater increase in IFN-γ mRNA expression upon treatment with MVC (**g**). DPV (**h**) and TDF (**i**) are shown in the bottom row. Relative fold change in expression was determined by setting vehicle control to 1. Data are plotted as mean ± SEM. Individual donors are depicted with specific black symbols.