Reciprocal modulation of antiretroviral drug and steroid receptor function *in vitro.*

Running title: ARV and steroid receptor cross-talk

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Millions of women are exposed simultaneously to antiretroviral drugs (ARVs) and progestin-based hormonal contraceptives. Yet, the reciprocal modulation by ARVs and progestins of their intracellular functions is relatively unexplored. We investigated the effects of tenofovir disoproxil fumarate (TDF) and dapivirine (DPV), alone and in the presence of select steroids and progestins, on cell viability, steroid-regulated immunomodulatory gene expression, activation of steroid receptors and anti-HIV-1 activity *in vitro*. Both TDF and DPV modulated the transcriptional efficacy of a glucocorticoid agonist via the glucocorticoid receptor (GR) in the U2OS cell line. In TZM-bl cells, DPV induced the expression of the pro-inflammatory interleukin (IL)-8 gene while TDF significantly increased medroxyprogesterone acetate (MPA)-induced expression of the anti-inflammatory glucocorticoid-induced leucine zipper (GILZ) gene. However, peripheral blood mononuclear cell (PBMC) and ectocervical explant cell viability and gene expression results along with TZM-bl HIV-1 infection data are reassuring and suggest that TDF and DPV, in combination with dexamethasone (DEX) or MPA, do not reciprocally modulate key biological effects in primary cells and tissue. However, we show for the first time that TDF induces progestogen-independent activation of the progesterone receptor (PR) in a cell line. The capacity of TDF and DPV to influence GR and PR activity suggests that their use may be associated with steroid receptor-mediated off-target effects. This, together with cell line and individual donor gene expression responses in the primary models, raises concerns that reciprocal modulation may cause side-effects in a cell- and donor-specific manner *in vivo*.

Introduction

Women in sub-Saharan Africa are disproportionally affected by the human immunodeficiency virus (HIV-1) and face a high risk of unintended pregnancy and sexually transmitted infections (1). As a result, millions of HIV-1 positive women are concurrently taking oral ARVs and progestogen-containing hormonal contraceptives. Multipurpose prevention technologies (MPTs) are in development to safely and effectively protect healthy women against HIV-1 and unwanted pregnancy. Understanding the relationship between ARVs and hormonal contraceptives is therefore essential to give insight on potential short- and long-term side effects associated with their simultaneous use. Adverse effects associated with ARV and hormonal contraceptive use include bone density loss, kidney and liver toxicity, cardiovascular damage, lactic acidosis and effects on immune function (2, 3). However, studies on the reciprocal modulation of ARV and synthetic progestogen (progestin) intracellular activity are limited. We hypothesize that progestins may affect ARV efficacy and that ARVs may modulate steroid-receptor activity in the absence and presence of steroids, which may impact on the efficacy and side-effects of these drugs.

Tenofovir disoproxil fumarate (TDF), the prodrug form of tenofovir (TFV), is a nucleotide reverse transcriptase inhibitor, widely used in combination with other ARVs in the HIV-1 treatment regimen (4). Currently, TDF is approved as an oral pre-exposure prophylaxis (PrEP) method in combination with emtricitabine, to prevent HIV-1 infection in healthy individuals. It has been shown to be safe and well-tolerated in Phase I clinical trials as an intravaginal ring in sexually abstinent women; however, a recent Phase I trial assessing the safety and pharmacokinetics of a ninety-day TDF intravaginal ring in sexually active women found that the ring causes ulcerations and increases inflammatory markers in women using the ring compared to placebo (5, 6). Dapivirine (DPV), a non-nucleoside reverse transcriptase

68 inhibitor, is the most clinically advanced microbicide targeted for intravaginal delivery. In
69 2016, two Phase III clinical trials found that a thirty-day DPV intravaginal ring reduced risk
70 of HIV-infection by approximately 30% and modelling data from follow up open label
71 extension studies have suggested a greater risk reduction of approximately 50% overall for
72 women using the ring over a one year period (7-9). The DPV ring is now under regulatory
73 review for use in developing countries with high HIV incidence.

74 The most commonly used progestin in sub-Saharan Africa is the intramuscular (IM), three-
75 monthly, injectable contraceptive medroxyprogesterone acetate (MPA), referred to as Depo-
76 Provera or depot MPA (DMPA-IM) (10). Levonorgestrel (LNG), widely used in the region as
77 a contraceptive in different formulations and delivery methods, is also the leading candidate
78 for the development of dual ARV/progestin MPTs. These progestins are synthetic steroid
79 ligands that elicit their intracellular responses by binding to and activating steroid receptors
80 (11). The primary targets for progestins are the progesterone receptors (PR-A and PR-B);
81 however, some progestins such as MPA are also known to cross-talk with other steroid
82 receptors, such as the glucocorticoid receptor (GR) and the androgen receptor (AR) (12).
83 Through their actions as ligand-activated transcription factors, steroid receptors can activate
84 or repress transcription to influence multiple processes in cells and tissues (13). MPA is a
85 known full to partial GR agonist and induces immunomodulatory effects and increased HIV
86 infection via GR-mediated mechanisms *in vitro* (13-15). Clinical epidemiological studies also
87 suggest that DMPA-IM increases HIV-1 risk relative to no contraception (16) and LNG
88 implant (17).

89 Several clinical and pharmacokinetic studies have evaluated the effects of simultaneous use
90 of some ARVs and progestins on pregnancy, HIV-1 acquisition or disease progression, and
91 changes in serum drug concentrations. To date, efavirenz (EFV) is the only ARV shown to

92 decrease contraceptive efficacy by influencing the metabolism of progestins in women,
93 resulting in a decrease in progestin levels (18, 19). Studies evaluating the effect of hormonal
94 contraceptives on ARV efficacy showed that DMPA-IM, LNG and combined oral
95 contraceptives have no detectable effect on the efficacy of combined antiretroviral therapy
96 containing non-nucleoside reverse transcriptase inhibitors other than DPV, or protease
97 inhibitors (20). Limited *in vitro* studies have shown that TDF and DPV have
98 immunomodulatory effects (21-23), and this may indicate the involvement of steroid
99 receptors such as the GR, a known immune regulator. A recent *in vitro* study showed that
100 MPA, but not LNG or norethisterone, is able to inhibit the anti-HIV-1 activity of TFV and
101 tenofovir alafenamide in blood and tissue CD4+ T cells, most likely via lowering intracellular
102 concentrations of TFV-diphosphate (24). These clinical and *in vitro* studies suggest that drug-
103 drug interactions do occur *in vivo.*

104 Potential off-target effects and drug-drug interactions of ARVs and progestins are likely to be
105 dose-dependent. Intravaginal and serum or plasma concentrations of progestins and ARVs
106 used for PrEP and MPTs exhibit a wide range of concentrations (Table 1). Additional *in vivo*
107 studies are required to investigate drug-drug interactions as well as *in vitro* studies to identify
108 and predict as yet unidentified off-target effects and their dose-dependency.

109 In the present study we evaluated the capacity of select steroid ligands and ARVs to
110 reciprocally modulate their respective intracellular functions; namely, the regulation of gene
111 expression via activation of steroid receptors by steroid ligands, and inhibition of HIV-1
112 infection by TDF and DPV. Dose-dependent effects of TDF and DPV on GR, PR and AR
113 activity in the absence and presence of receptor agonists in *in vitro* cell line, PBMC and
114 ectocervical tissue explant models were investigated and effects of MPA, LNG and other
115 receptor-specific agonists on the dose-dependent inhibition of HIV-1 infection by TDF and
116 DPV were investigated in the TZM-bl reporter cell line.

Materials and Methods

Ethics

Ethical approval to conduct this study was granted by the Human Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town, South Africa (approval number: HREC 210/2011).

Test compounds

The ARVs, TDF and DPV were purchased from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (USA) and Selleck Chemicals (USA) respectively. The ARVs were made up to a stock concentration of 10^{-1} M in dimethyl sulfoxide (DMSO, Sigma-Aldrich, South Africa). DMSO was chosen as the solvent due to its high maximal solubility of approximately 10^{-1} M for both TDF and DPV (25, 26). Stock concentrations of ARVs were serially diluted 1:10 with DMSO from 10^{-2} M to 10^{-7} M. The steroids used in the study included the progestins, MPA, and LNG as well as synthetic GR and AR agonists, DEX and mibolerone (MIB), respectively. These were obtained from Sigma-Aldrich (South Africa), except for MIB, which was obtained from Perkin Elmer (USA). Stock concentrations of steroids ranged from 10^{-3} M to 10^{-6} M in ethanol (EtOH). Ligands or control vehicle were added to cells to give final concentrations as indicated in the figures, such that all incubations contained 0.1% (*v*/*v*) EtOH and 0.1% (*v*/*v*) DMSO. The use of EtOH and DMSO as vehicle at these low final concentrations is common practice in the literature and was not toxic to the cells (27-29).

Cell culture

Human cervical indicator TZM-bl cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) were used for the HIV-1 inhibition assays. U2OS human osteosarcoma cells

were used for the luciferase reporter assays as they are deficient in endogenous steroid receptors (American Type Culture Collection (ATCC), USA). MDA-MB-231 cells stably transfected with PR-B were a kind gift from Professor Valerie Lin (Nanyang Technological University, Singapore) and these were also used for luciferase reporter assays. HEK293T human embryonic kidney cells (ATCC, USA) were used to generate infectious molecular clones. All cells, except the MDA-MB-231 breast cancer cells, were grown in 75 cm^2 flasks in full 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 calf serum (FCS) (Thermo Scientific, South Africa), 100 IU/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, South Africa). MDA-MB-231 cells were cultured in full DMEM supplemented with 7.5% (*v/v*) FCS (Thermo Scientific, South Africa), and 100 mg/mL neomycin (Sigma-Aldrich, South Africa). For experimental incubations with MDA-MB-231 cells, phenol red-free full DMEM supplemented with 5% charcoal stripped (c-s) FCS was used. Cells were maintained at 37°C in a water-jacketed incubator (90% humidity and 5% CO_2).

Cell Viability

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, South Africa) according to the manufacturer's instructions and measured on a spectrophotometer (Thermo Scientific, USA) at 595 nm. DMSO was used as the positive control for cytotoxicity at concentrations ranging from 5 – 20 % (30, 31).

PBMC isolation

Whole blood from anonymous healthy female donors who were negative for HIV-1, syphilis and hepatitis B and C was obtained from the Western Cape Blood Service (South Africa),

after written informed consent. PBMCs were isolated using Histopaque (H1077 Hybri-MaxTM; Sigma-Aldrich, South Africa) density centrifugation with Leucosep tubes (Greiner Bio-One, Germany) according to the manufacturer's instructions. PBMCs were isolated as previously described (15). Cells were incubated overnight, and thereafter pelleted and washed twice by centrifugation at 250 × *g* in 1 × PBS supplemented with 1% (*v/v*) c-s FCS.

Cervical tissue explants

Cervical tissue was obtained from seven HIV-1 negative, pre-menopausal women, with a normal pap smear and undergoing hysterectomies for benign reasons, after informed consent. Anonymised fresh tissue was supplied from two sites in the Western Cape, South Africa; namely, Groote Schuur Hospital and Tygerberg Hospital. The majority of the samples were positive for HSV-1 and negative for HSV-2. Cervical tissue was processed as previously described (14) between 1 to 3 hours post-operation.

Stimulation with compounds, RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

TZM-bl cells were seeded at a concentration of 1×10^5 cells/mL in 12-well plates in full DMEM. After 24 hours, cells were stimulated with ARVs or steroids for 24 hours and thereafter harvested in 400 µL TriReagent® (Sigma-Aldrich, South Africa). PBMCs 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 and thereafter pelleted by centrifugation at 250 × *g* for 5 min and harvested in 400 µL TriReagent® (Sigma-Aldrich, South Africa). TZM-bl cells and PBMCs were then processed for RNA according to the manufacturer's instructions. Cervical tissue explants were stimulated in triplicate or quadruplicate with steroid ligands and ARVs in full

RPMI and incubated at 37°C in a water-jacketed incubator (90% humidity and 5% CO2) for 48 hours. For RNA isolation, cervical explants were harvested in 800 µL QIAzol® in 2 mL cryovial tubes (Nunc, Germany) and were subsequently homogenized using a hand-held homogeniser (TissueRuptor®, Qiagen, The Netherlands) with disposable probes (TissueRuptor® Probes, Qiagen, The Netherlands). Cervical explant tissue RNA was thereafter isolated using the RNeasy® Microarray Tissue Mini Kit (Qiagen, The Netherlands), according to the manufacturer's instructions. Times chosen for incubation of compounds in the different models were based on previous experiments in our laboratory showing that robust changes in gene expression occurred at these time points. Whether different effects occurred after different times was not investigated.

250 ng RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, South Africa), according to the manufacturer's instructions. Real-time qRT-PCR was performed using the FastStart Essential DNA Green Master kit (Roche) on a RotorGene 3000 (Qiagen, The Netherlands) qRT-PCR machine, according to the manufacturer's instructions. The genes investigated were the anti-inflammatory GILZ gene, and the pro-inflammatory IL-8 and IL-6 genes. GILZ is a validated primer set purchased from Qiagen South Africa. IL-6 and IL-8 primers have previously been reported (32). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene as previously reported (33). The IL-8 and GAPDH primers were used at a concentration of 500 nM, while IL-6 primers were used at 250 nM. The qRT-PCR profiles for IL-6, IL-8 and GAPDH were established by Verhoog *et al.* (33).

Virus propagation and TZM-bl infection assay

Initial viral stocks were prepared as previously described. TZM-bl cells were seeded at a concentration of 5×10^4 cells/mL in a 96-well flat-bottomed culture plate in full DMEM. The

following day the TZM-bl cells were either stimulated with progestin or its vehicle control (0.1% *v/v* EtOH) and/or ARV or its vehicle control (0.1% *v/v* DMSO) for 24 hours in triplicate. Cells were then infected with 20 IU/mL HIV-$1_{BaL\text{-}Renilla}$ and were harvested 48 hours later with Bright-Glo luciferase lysis buffer (Promega, USA). Luminescence was determined on a luminometer (Modulus Microplate, Promega, USA) in which relative light units were measured for each well. Cell viability was measured using the MTT assay by measuring absorbance at 595 nm on a spectrophotometer (Thermo Scientific, USA). Luciferase readings were normalized to MTT values (RLU/MTT). Dose-response data were analyzed relative to the maximal response generated by the vehicle (set to 100%) and a non-linear regression model was employed with the Hill slope set to unity. IC_{50} values were compared using an unpaired, two-tailed *t*-test.

Plasmids and transfection

U2OS cells were transfected with either the GR (34), AR (35) or the PR (36) reporter systems each containing the steroid receptor plasmid, a luciferase reporter gene plasmid (pTAT-GRE-LUC, (37) containing the receptor response elements and an empty vector (pCDNA3, Invitrogen, UK) that was used as the negative control for the steroid receptor plasmid. To ensure consistent transfection efficiency, cells were transfected in a $10cm^2$ dish (Greiner Bio-one International) at a density of 1.5×10^6 cells using X-tremeGENE 9 (Roche, South Africa) according to the manufacturer's recommendations. Thereafter the cells were trypsinized, replated into 96 well plates and stimulated with compounds for 24 hours. The transfection conditions for each receptor were as follows: Human GR plasmid (10 µg pcDNA3-hGR + 3.75 µg pTAT-GRE-LUC), human AR (2.5 µg pSV-hAR + 1.88 µg pTAT-GRE-LUC), and human PR-B (3.5 µg pSG5-hPR-B + 1.41 µg pTAT-GRE-LUC). MDA-MB-231 cells, that are stably transfected with PR-B, were only transfected with 9 µg pTAT-GRE-LUC.

Luciferase reporter assay

The U2OS cells were incubated with the respective ARVs and/or steroid treatments for 24 hours. Thereafter, the cells were washed with ice-cold PBS and harvested in 25 µL reporter lysis buffer (Promega, Madison, WI, USA). The luciferase activity for each condition was detected in the presence of the substrate luciferin (Promega, Madison, WI, USA) using a Modulus Microplate luminometer and was normalized to total protein concentration as determined by the Bradford assay (38).

Western blotting

To confirm steroid receptor transfection, western blot analysis was performed, essentially as previously described (39), from lysates of U2OS cells bulk-transfected with GR, PR or AR expression vectors or the empty vector pCDNA3 in luciferase reporter assays. Positive controls for each receptor were prepared from COS-1 cells seeded in 12-well plates at a density of 1×10^5 cells/mL and, after 24 hours, transfected with 1 µg GR, PR or AR expression vectors. Transfected U2OS cells were seeded in 12-well plates at a density of 1×10^5 cells/mL. The next day cells were washed once in PBS and 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 minutes. The following antibodies were used: anti-AR 1:1 000 (441; sc-7305), anti-GR 1:5 000 (G-5; sc-393232), anti-PR 1:1 000 (NCL-L-PGR-312, Leica Biosystems), and anti-GAPDH 1:15 000 (0411; sc-47724). Goat anti-rabbit secondary antibody (sc-2313) was used for the anti-AR antibody, at a 1:10 000 dilution and an anti-mouse secondary antibody (m-IgGκ BP-HRP: sc-516102) was used for the antibodies to GR, PR and GAPDH and was added at a 1:5 000 dilution.

In silico molecular docking

All computational predictions were carried out using desktop workstations running the Scientific Linux 7.4 OS using the Glide utility included in the Schrodinger 2017-3 suite release. Protein Data Bank entries of the steroid receptors (1E3G, 1XQ3, 3D90, 4UDC) were prepared using the Maestro PrepWizard. Structures were completed with the addition of bond orders and missing side-chains. Non-bound waters were removed and, where applicable, the B chain of dimerized structures was removed. Automated optimization protocols were then run to refine the structures. Glide docking grids of default length were created centred on the native ligands. Docking simulations were performed iteratively using the Glide SP setting until a plausible docking pose was found. Binding energies were calculated using the Prime MM-GBSA minimization and the binding energy calculation package provided with the Schrodinger suite. MM-GBSA calculations were performed using the variable-dielectric generalized Born solvent model. The minimization was performed with flexibility tolerated for all protein atoms within a 10 Å radius of the ligand.

Data analysis

Results were analyzed using GraphPad Prism (version 7) software from GraphPad Software Inc. (La Jolla California, USA). For dose-response curves, the receptor agonists were used as reference ligands and set to 100%. Dose-response curves were fit with a non-linear regression model using “log agonist vs response”, with a fixed Hill slope of one, to obtain the best-fit maximal responses. All other curves were then plotted relative to the best-fit maximal value of the reference ligand. All the data were tested for normality and parametric or non-parametric tests were performed accordingly (40). Unpaired *t*-tests were performed to compare the EC_{50} values and maximal responses of dose-response curves from different treatments. For experiments that had one condition or two different conditions, a parametric

one-way or a two-way ANOVA was performed with a Tukey multiple comparison post-test (comparing all groups to the vehicle control) or a non-parametric Kruskal-Wallis test was performed with a Dunn's multiple comparison post-test. Data were plotted as mean ± SEM on histograms, with the number of replicates per condition and the number of independent biological repeats (n values) described in each figure legend.

Results

The concentration ranges of ARVs used in this study were selected to reflect the range of concentrations that have previously been measured intravaginally and in blood (Table 1). Steroid concentrations were chosen based on concentrations intended to fully saturate possible cognate steroid receptors or, for dose-response analysis, spanning pharmacologically-relevant concentrations of steroids (13, 27, 41). Steroid stimulation times of 24 or 48 hr were used as these have been previously established to elicit inflammatory gene responses in the models used in the study.

Effects of ARVs and steroids on cell and tissue viability

We investigated whether ARVs affect cell or tissue viability using MTT assays. In TZM-bl cells, concentrations of TDF ranging from 0.01 to 100 µM and concentrations of DPV ranging from 0.01 to 1 µM did not affect cell viability (Fig. 1A-B). However, 10 and 100 µM DPV significantly reduced cell viability (Fig. 1A-B). In PBMCs, only 100 µM of both ARVs significantly reduced cell viability (Fig. 1C-D). A concentration of 5% DMSO was used as a positive control for cytotoxicity in TZM-bl cells (Fig. 1A-B) and a range of 5-20% DMSO was used in PBMCs (data not shown). Tissue viability of ectocervical explants appeared unaffected by TDF and DPV concentrations ranging from 1 to 100 µM (Fig. 1E-F). Cell viability of ARVs was not altered by the presence of 100 nM steroids DEX, or MPA, alone or in combination with 1 µM of the ARVs in TZM-bl cells However; in PBMCs, the presence of

100 nM DEX significantly reduced cell viability by approximately 30% (Fig. S1A-B). We next performed gene expression analysis in the various models using only ARV concentrations shown not to reduce viability of the cell/tissue models, to avoid confounding the interpretation of the data. Given the slight reduction in cell viability by 100 nM DEX in PBMCs, all the PBMC gene expression results were normalized for cell viability and interpreted with due caution.

ARV modulation of endogenous steroid-regulated inflammatory genes

To examine the capacity of ARVs to modulate endogenous immunomodulatory genes, relative mRNA expression of the established (42-44) steroid-regulated anti-inflammatory gene GILZ and two pro-inflammatory genes, IL-6 and IL-8 were assessed in TZM-bl cells, PBMCs and tissue explants. These genes were chosen as model GR-regulated genes. GILZ has previously been shown to be upregulated in several model systems by glucocorticoids via a mechanism involving binding of the GR to glucocorticoid-response elements in the promoter (45). IL-6 has previously been shown to be downregulated in several model systems by glucocorticoids and other steroids via a mechanism involving tethering of the steroid receptor to other transcription factors such as NFκB, recruited to the promoters (46), while effects on IL-8 are reportedly variable (33, 47). Cells and tissues were stimulated with 1 μM DPV or 10 μM TDF in the absence and presence of 100 nM DEX, or MPA. In all three model systems, DEX and/or MPA was found to increase GILZ and repress IL-6 mRNA levels as expected, while no statistically significant effects were observed for IL-8. The results are summarized in Table S1.

In TZM-bl cells, neither TDF (up to 10 μM) nor DPV (up to 100 nM) affected GILZ mRNA levels on their own (Fig. S2A and Fig. S3A, B). However, TDF, more so than DPV, increased the efficacy of MPA-induced GILZ expression in MPA dose-response analysis,

although these effects were not statistically significant (Fig. 2A-B). TDF dose response analysis (1 to 100 µM TDF), in the presence of 100 nM MPA, revealed that TDF at 10 and 100 µM significantly increased MPA-induced GILZ expression by approximately 1.5 and 2-fold, respectively (Fig. S2A) but had no significant effect on the MPA response for IL-6 and IL-8 in TZM-bl cells (Fig. S2B-C). Unlike TDF, DPV alone significantly induced IL-8 mRNA expression by 11-fold and appeared to induce IL-6 mRNA by 10-fold in TZM-bl cells (Fig. 2C-D). DEX and MPA significantly repressed IL-6 expression by 10-fold and 5-fold respectively and appeared to induce IL-8 expression by 3.4-fold and 3.5-fold, respectively (Fig. 2C-D). Co-stimulation with TDF did not appear to alter these DEX or MPA responses (Fig. 2C-D). However, co-stimulation with DPV inhibited DEX and MPA repression and returned IL-6 expression to basal levels (Fig. 2C). Interestingly, for IL-8 expression the effect of the combination of DPV with DEX and MPA appeared to be steroid-specific. DEX in combination with DPV did not alter the DPV-induced IL-8 expression; however, the combination of MPA and DPV resulted in a significant 1.6-fold potentiation of the DPV-induced IL-8 response (Fig. 2D). Lower doses of TDF and DPV in the range of 0.1-100 nM, had no significant effect on the mRNA levels of GILZ, IL-6 and IL-8, both in the absence and presence of MPA (Fig. S3).

Results in PBMCs are shown for pooled effects on 13 donors (Fig. 3) as well as for subgroups of donors to illustrate more clearly the diversity and frequency of donor-specific effects (Figs. S4-S6). In PBMCs TDF and DPV had no detectable effect on GILZ mRNA levels in the majority of donors (Fig. 3A and Fig. S4A). As expected, DEX treatment significantly induced GILZ mRNA expression by 8-fold, as for TZM-bl cells, but this remained unchanged by the presence of ARVs, unlike in TZM-bl cells. As expected, DEX also significantly repressed IL-6 mRNA levels by about 3-fold, which was not influenced by combination with ARVs (Fig. 3B). DEX alone and in combination with ARVs had no

detectable effect on IL-8 mRNA levels (Fig. 3C). In the majority of donors, DPV and TDF showed no detectable significant effects on IL-6 and IL-8 mRNA expression (Fig. 3B-C, Fig. S5B, and Fig. S6B). The PBMC responses showed a high degree of variability in donor sample responses. Subgroup analysis did, however, reveal donor-specific effects, with some donors exhibiting at least a 2-fold up-regulation (as for IL-6 and IL-8 in TZM-bl cells) or down-regulation, or changes of less than 2-fold in mRNA levels of some genes in response to ARVs (Fig. S5 and Fig. S6).

Experiments in explant tissue were performed with DPV and not TDF, due to limited availability of fresh tissue. Most of the results were very similar to those obtained in PBMCs (Fig. 4A-C). DPV alone appeared to have no effect on GILZ mRNA (Fig. 4A). As observed in PBMCs or TZM-bl cells, DEX significantly induced GILZ mRNA by 8-fold, and MPA also appeared to increase GILZ mRNA by 2.4-fold (Fig. 4A). MPA and DEX alone appeared to repress IL-6 by approximately 2 and 1.5-fold, respectively, but had no effect on IL-8 (Fig. 4C), as observed in PBMCs and TZM-bl cells. DPV did not appear to modulate the DEX or MPA responses for any of the genes (Fig. 4A-C). As for PBMCs, there was high donor-specific variability in responses, making it difficult to establish significance for small effects. While DPV alone appeared to have no effect on all three genes for the pooled samples, it appeared to have a pro-inflammatory effect on IL-6 and IL-8 for some of the donor samples (Fig. 4B-C), as for some PBMC donor samples and in TZM-bl cells.

ARV effect on steroid receptor transcriptional activity

We next investigated the effects of ARVs on the efficacy and potency of the transcriptional effects of GR, PR-B and AR receptor agonists by using promoter-reporter assays. Western blotting showed that steroid receptors were successfully exogenously expressed in U2OS cells (Fig. S7). *In vitro* toxicity of TDF and DPV in U2OS cells, as assessed via the MTT cell

viability assay, showed that concentrations up to 1 µM for both ARVs were not deleterious, while 10 µM DPV but not TDF resulted in loss of viability (Fig. S8).

TDF (1 µM) significantly increased the efficacy of DEX transcriptional activity via the GR by 33%; DPV had a similar effect, which approached significance (p=0.0531), increasing DEX transcriptional activity by 25% (Fig. 5A-B and Table S2), similar to the effect of MPA in TZM-bl cells (Fig.2A-B, Fig. S2A). The potency of DEX was not significantly altered in the presence of either ARV (Table S2). Neither ARV appeared to have a significant effect on the efficacy and the potency of the AR synthetic agonist MIB (Fig. 5C-D and Table S3). Surprisingly, 1 µM TDF, unlike DPV, significantly increased PR-B-mediated transcriptional activity alone and in the presence of low concentrations of LNG (0.0001 nM to 0.001 nM) (Fig. 5E). At higher concentrations of LNG, TDF did not significantly affect the efficacy of LNG via the PR. DPV also had no effect on the transcriptional efficacy and potency of LNG via the PR (Fig. 5F and Table S4). Potencies and efficacies for each of the dose-response curves in Fig. 5 are detailed in Table S2-4.

Following the observation that TDF transactivated the PR-B in a progestogen-independent manner, the dose-dependency of this response was similarly assessed using a promoter-reporter assay in U2OS cells. The cells were stimulated with increasing concentrations of TDF ranging from 1 nM to 1 µM in the absence and presence of 100 nM progesterone (P_4). TDF had an EC_{50} of 599 ± 33 nM for the PR-B and the potency was significantly decreased in the presence of 100 nM P_4 (Fig. 6 and Table S5). Agonist-independent effects of TDF and DPV at concentrations ranging from 100 nM to 10 µM via the AR and GR were also assessed, but no significant induction of these receptors in the absence of receptor agonists was observed (Fig. S9).

Similarly, the induction of PR-B transcriptional activity by TDF was investigated in the MDA-MB-231 cell line that stably and constitutively expresses PR-B (Fig. 7 and Table S6). As expected, 1 µM TDF activated PR-B in the absence of LNG (Fig. 7) and significantly increased the transcriptional efficacy of LNG in these cells (Table S6). As shown in U2OS cells, DPV did not influence the transcriptional efficacy or potency of LNG in MDA-MB-231 cells.

The data observed in the current study reveal that TDF, but not DPV, significantly affects GR and PR steroid receptor function, while both TDF and DPV have no significant effects on the AR. These differential activities may result from direct interaction between the ARVs and the steroid receptors. To investigate this *in silico* molecular docking was employed to assess whether TDF and DPV exhibited an affinity for the ligand-binding pockets of either the GR, PR-B or the AR. (For this latter receptor, two structures were evaluated.) To validate these calculations, binding affinities were first predicted for the crystallized agonist in complex with the receptor ligand-binding domain of each receptor. Following these test simulations, the results suggested that TDF had low affinity for the ligand-binding pockets of all three steroid receptors compared to the receptor agonists (Table S7). DPV exhibited some binding affinity for the PR and GR, although this was not reflected in transcriptional activity in which DPV had no significant effect on GR or PR activity on its own. Mono- and di-anionic charged states of TFV were also evaluated, and these species exhibited negligible affinity for the ligand binding domains of the three receptors.

Modulation of ARV efficacy by steroids

The effects of steroid ligands on the efficacy and potency of ARVs to inhibit HIV-1 viral replication was assessed using HIV-1 infection assays in TZM-bl cells. Ligands were chosen that bind to either the GR (DEX), the AR (MIB, LNG) or both the AR and GR (MPA) (27,

41, 44, 48). DEX and MPA alone appeared to increase HIV-1 infection, as previously reported (15), although in this experiment the effect was not statistically significant (Fig. 8A-B). We found that 100 nM DEX, MPA, LNG and 0.01 nM MIB did not significantly affect the efficacy or potency of TDF and DPV to inhibit HIV-1 infection (Fig. 8).

Discussion

In the present study we evaluated the capability of TDF and DPV to reciprocally modulate their respective intracellular functions alone and together with steroid ligands.

In TZM-bl cells, PBMCs and explant tissue DEX or MPA alone exhibited anti-inflammatory effects by upregulation of GILZ and downregulation of IL-6 mRNA. These results provide confidence that detection of expected steroid-induced changes in gene expression in these models was reproducible. Differential regulation of select immune function genes by TDF and DPV in the absence and presence of steroid ligands was observed in TZM-bl cells. However, no effects were detected for the genes assessed in pooled data from PBMCs and explant tissue. In TZM-bl cells DPV induced pro-inflammatory effects on IL-6 and IL-8 genes, unlike TDF. High concentrations of TDF and DPV were found to increase MPA-induced efficacy on GILZ in TZM-bl cells. Similar effects were observed for DEX-induced efficacy on a promoter-reporter plasmid in U2OS cells. DPV effects were modulated on IL-6 and IL-8 genes by DEX or MPA in TZM-bl cells, and this was not seen for TDF. DEX and MPA inhibited the DPV-induced pro-inflammatory response on the IL-6 gene. MPA also enhanced the proinflammatory effect of DPV on IL-8 in TZM-bl cells, an effect not observed for DEX. In contrast, the results in PBMCs and explants are reassuring in that they suggest, if they occur *in vivo*, that there is very little reciprocal modulation of immunomodulatory gene expression for these ARVs and clinically relevant steroids. However, the cell line results

provide proof of concept that reciprocal modulation could occur in some cells, including both ARV- and steroid-specific effects, but their physiological relevance is unclear.

The results for both PBMCs and explants show a high degree of inter-individual donor variability, as reported previously (49, 50). Some of the individual donor responses we observed in the primary models correspond to effects observed in TZM-bl cells and raise the possibility that for some donors, ARVs may have both pro-inflammatory and anti-inflammatory effects and some reciprocal modulation between these ARVs and steroids could occur.

These donor-specific effects on gene expression in primary models may be reflected in the varied responses of patients to ARV treatment, the underlying factors for which may include patient genotype, age, endogenous hormone and other drug levels, nutrition and disease state (51-53). About 60% of the explant tissue donors were positive for HSV-1 and had a varying age range between 31–56 years which may contribute to the varied responses observed. Female donors for PBMCs were negative for some infections including HIV-1 and syphilis but were not tested for all possible infections or HSV-1 infection and thus it is unknown whether other underlying infections influenced the immune gene responses to ARVs.

The immunomodulatory effects we observed with TDF (0.1 nM – 100 µM) and DPV (0.1 – 1 µM) in TZM-bl cells in the present study occur at physiologically-relevant ARV doses (TDF: 1.2 nM-79 µM; DPV: 0.7 nM-21 µM) (Table 1). We did not detect responses on select genes *in vitro* in PBMCs with 1 µM TDF or DPV, concentrations greater than those detected (0.7-5 nM) in serum of patients, suggesting that these ARVs alone are unlikely to have systemic immunomodulatory effects in vivo. However, it is possible that effects on PBMCs may be observed at lower ARV concentrations. We also did not detect responses on select genes *in vitro* in ectocervical tissue with 1 µM DPV alone, suggesting that inflammatory effects do not

473 occur at this concentration. However, this may occur at higher FGT concentrations, as
474 measured for the DPV vaginal ring (Table 1). *In vivo* clinical data of the DPV vaginal ring
475 suggest this is unlikely as no serious adverse effects of the ring indicating excessive
476 inflammation in the genital tract of women who used the ring in Phase II and III clinical trials
477 were detected (9, 54). Phase 1 clinical trials of the TDF vaginal ring in sexually abstinent
478 women have previously reported that it was safe with minimal adverse events. However, a
479 recent Phase 1 clinical trial showed that women using the TDF ring compared to women
480 using a placebo ring had higher expression of cytokines and chemokines including IL-6 and
481 IL-8 (5, 6). Further experiments are required to investigate immunomodulatory effects of
482 high doses of DPV and TDF in FGT tissue models.

483 Our data in TZM-bl cells contribute to the limited *in vitro* studies that show that ARVs
484 influence immune function independently of their HIV-1 inhibitory action.
485 Immunomodulatory effects of TDF have previously been described in human PBMCs. After
486 infection with live bacteria, followed by TLR or TNF-α- stimulation, TDF at doses ranging
487 from 12.5 – 50 μM decreased expression of the pro-inflammatory cytokine IL-8 and the anti-
488 inflammatory cytokine IL-10 and increased expression of the pro-inflammatory cytokine IL-
489 12 (28). TFV at a concentration of 3.5 mM has been shown to upregulate the expression of
490 MIP-3α, IL-8 and TNF-α in macrophages derived from blood monocytes, as well as in
491 primary epithelial cells derived from the endometrium and ectocervix of healthy women (21,
492 22). However, these reports (21, 22, 28) used much higher concentrations of these ARVs
493 than are detected systemically. Additionally, in human genital epithelial cells, DPV at
494 concentrations of 10 μM and above was shown to produce small increases in IL-8 secretion
495 (23), consistent with our findings in TZM-bl cells. Our results further suggest that side-
496 effects of TDF and DPV due to loss of cell viability are unlikely to occur at concentrations up

to 10 µM for both ARVs in PBMCs and 100 µM in ectocervical tissue, although this is likely to be cell- and tissue-specific.

To our knowledge, this is the first study to investigate the effects of ARVs on GR, PR and AR transcriptional activity in the presence of their respective agonists. Svard *et al.* previously reported that a panel of ARVs, including TFV and EFV, bind to the liver X receptor (LXR) and the estrogen receptor (ER). In the study by Svard *et al.*, ARVs predicted to bind to the GR were not able to bind *in vitro* or to transcriptionally activate the GR (55). Our results show that TDF and DPV alone do not affect the transcriptional activity of the GR (Fig. S9). We show for the first time that TDF and DPV increase the transcriptional efficacy of the GR agonist DEX in U2OS cells and that TDF significantly activates the PR in a ligand-independent manner. In contrast, TDF and DPV had no effect on the potency or efficacy of the AR agonist MIB.

These *in vitro* ARV effects on GR and PR activity are potentially important if they are translated *in vivo*. For ubiquitously expressed GR, our results suggest that, *in vivo*, TDF and DPV may potentiate the effects of GR ligands, such as when cortisol levels are high or during glucocorticoid therapy. This may be relevant for several GR-regulated physiological processes, including metabolism, bone mineral density (BMD) and cardiovascular and immune function (56). BMD loss is a known side-effect of TFV use in HIV-1-positive patients and in HIV-1-negative PrEP users (57, 58). Several *in vitro* studies have implicated TDF in playing a role in decreasing BMD (59, 60). Activation of the GR has also been implicated in decreasing BMD (61, 62). Whether the GR is involved in any of the above effects of TDF is unknown. Since the progestin MPA is a potent partial to full agonist of the GR (63), HIV-1 positive women using DMPA-IM for contraception and taking TDF or DPV

may exhibit increased GR activity to increase side-effects, such a loss of BMD or immunosuppression, and/or other beneficial effects of GR-regulated physiological functions.

A novel finding demonstrated in this study is that TDF, but not DPV, significantly activates the transcriptional activity of the PR-B in the absence of a progestogen and increases the efficacy of the PR-B in the presence of the progestogen LNG. This has important potential implications that may be relevant to PR-regulated physiological processes including reproduction, reproductive tissue cancers (64), immune function and bone density. TDF or TFV have not previously been shown to affect reproductive functions (65, 66). BMD is regulated by both estrogen and P_4 (67-69). DMPA-IM has also been shown to have a dose-dependent relationship with BMD loss in adolescent girls receiving high-dose intramuscular DMPA-IM (150 mg) compared to the lower dose subcutaneous DMPA-SC (104 mg) (67-71). These studies suggest that high concentrations of PR ligands such as MPA and LNG may create a hypoestrogenic environment and reduce BMD. Our results suggest a possible link between TDF use, the PR and/or the GR, and BMD loss. Since TDF use as an intravaginal ring for PrEP is set to deliver concentrations as high as 30 µM to the FGT (6), where the GR and PR are abundantly expressed (unpublished data, (72)), our results suggest that side-effects with TDF and/or DPV via the GR or PR in the FGT may be highly relevant. PBMCs are also shown to express GR mRNA and protein (73, 74) and some studies (73) but not others (74) show that they express detectable PR. This suggests that side-effects with TDF and/or DPV via the GR or PR may also be highly relevant in a systemic context.

The mechanisms whereby TDF but not DPV affect PR-B function remain to be determined and were beyond the scope of the present study. Our *in silico* docking data suggest that this is unlikely due to direct binding of TDF to the PR-B ligand-binding pocket. The finding that TDF alone does not cause similar activation of GR or AR, suggests the mechanism is specific

544 to PR-B signaling and is unlikely to involve general components of the transcriptional
545 machinery. A mechanism involving a direct effect on the PR-B, other than binding to the
546 ligand-binding pocket, or an indirect effect such as on a protein that interacts specifically
547 with the PR-B, may be involved. Further studies on transactivation and transrepression of
548 endogenous genes and more detailed mechanistic studies may provide further insights.

549 An encouraging finding of our study was that neither MPA nor LNG affected the potency or
550 the efficacy of TDF or DPV to inhibit HIV-$1_{BaL\text{-}Renilla}$ replication in TZM-bl cells. We have
551 previously also shown that this is the case for MPA and maraviroc (15), suggesting that these
552 progestins, when used for contraception, are unlikely to affect the efficacy for HIV-1
553 inhibition of these ARVs in women. This is consistent with previous clinical studies that
554 show that MPA does not affect ARV efficacy (20, 75). In contrast, a recent *in vitro* study
555 showed that MPA, but not LNG or NET, inhibited the anti-HIV-1 activity of TFV, in blood
556 and endometrial CD4+ T cells, most likely via lowering the intracellular TFV-diphosphate
557 levels (24). This suggests that the influence of MPA on ARV efficacy and potency may vary
558 depending on the cell type. A previous clinical study has also shown that TDF levels are
559 lowered in pregnant HIV negative women also suggesting that changes in hormones during
560 pregnancy may influence ARV efficacy (76). Previous clinical studies have not shown any
561 interactions of LNG with ARVs and hence LNG may be the more suitable choice for use as a
562 hormonal contraceptive in MPTs (77, 78).

563 Our study had several limitations including the limited donor size of PBMC and explant
564 models, which impacted on the statistical power of the study as well as on our ability to fully
565 characterize the effects of both ARVs and the progestins on inflammatory gene expression.
566 Furthermore, our gene expression analysis was restricted to three genes and models, and we
567 may have observed different results for others. Since we did not investigate effects of

concentrations of TDF and DPV greater than 1 µM in PBMCs, or DPV in genital tract tissue, it is possible that effects do occur *in vitro* under these conditions not investigated. Furthermore, we cannot draw any conclusions about *in vitro* effects in other genital tract compartments. The use of a transactivation model only of steroid receptor transcriptional activity is also a limiting factor as steroid receptor activity also occurs via a transrepression model, and the effect of ARVs on transrepression is unknown. The physiological relevance of the TZM-bl results remain to be explored i*n vivo*. Nevertheless, our findings provide novel insights into the mechanisms and reciprocal modulation of activities with combinatorial usage of ARVs and GR, AR and PR ligands, which may have important implications *in vivo*. Our PBMC and explant gene expression results and HIV-1 infection data are largely reassuring that TDF and DPV and DEX and MPA do not reciprocally modulate key biological effects and these ARVs would be suitable for combination in MPT with MPA. However, the TZM-bl gene expression data, individual gene expression responses for some donors in the primary models and effects of the ARVs on GR and PR function in vitro raise concern that some negative effects could occur in a cell- and donor-specific manner *in vivo*. Further experiments are required to evaluate dose- and time-dependent effects of ARVs and steroid receptor ligands *in vitro* and their physiological relevance *in vivo*.

Author Contributions Statement

S.D, M.K, and K.E performed about 25%, 20% and 15% of the experiments, respectively. S.S, J.G.W, J.M.M, A.B and M.F.M each contributed 8% of the experiments performed. All the authors contributed to design and planning of experiments and analysis and interpretation of data and contributed to the writing of the paper. J.P.H. conceived the project and directed the research. M.F.M. and C.A. played a significant role in co-supervision of S.D., M.K, and

S.S., K.E., respectively. J.P.H and S.D wrote most of the paper, with significant contributions by M.K. and J.G.W.

Acknowledgements

This work was supported by the Eunice Kennedy Shriver National Institute of Child Health & Human Development [R01HD83026] to J.P.H. Bursaries or fellowships were funded by the Harry Crossley Research Foundation (S.D, S.S), University of Cape Town (S.D, S.S, K.E, M.K), Poliomyelitis Research Foundation (K.E, M.K), Claude Leon Foundation (MFM) and the National Research Foundation of South Africa (S.S, K.E, M.K, J.G.W). The funders had no role in the study design, data collection or interpretation of the data from the study. The authors thank the following people for consenting suitable patients and providing cervical tissue: Shane Moore, Lynn Keck, Anne Hoffman and Tony Wu at Groote Schuur Hospital and Hennie Botha, Rudolf Boshoff and the registrars at Tygerberg Hospital. Lastly, the authors thank all the members of the Hapgood laboratory for intellectual discussions.

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Figure Legends

Figure 1. Effect of ARVs on cell viability in TZM-bl cells, PBMCs, and cervical explant tissue. TZM-bl cells (A-B), PBMCs (C-D), and ectocervical explant tissue (E-F) were treated with different concentrations of TDF and DPV, ranging from 10^{-10} M to 10^{-4} M, in triplicate, for 24 h and DMSO (0.1% *v/v*) was used as the vehicle control. 5% DMSO was used as the positive control in TZM-bl cells (Fig. S1) and in PBMCs (data not shown). Cells were treated with MTT reagent for 2 h and, after solubilisation, the absorbance was read at 595 nm. Cell viability was normalized to the vehicle control, which was set to 100%. Graphs show pooled results of three independent experiments for TZM-bl cells (A-B), data from four PBMC donors, with each condition done in triplicate and represented as mean ± SD (C-D) or pooled results from four cervical explant donors (E-F). Statistical analysis was done using a one-way ANOVA, followed by Tukey's multiple comparison post-test. Statistical significance is denoted by ** and *** to indicate $p < 0.01$ and $p < 0.001$ when comparing the vehicle control to ARV treated conditions.

Figure 2: Effect of TDF and DPV on inflammatory gene mRNA levels in human cervical TZM-bl cells. Gene expression was evaluated after 24 h stimulation with concentrations of ARVs and steroids as indicated. A-B: GILZ mRNA expression with an increasing dose of

MPA, in the absence and presence of 10 µM TDF (A) and 1 µM DPV (B). C-D: IL-6 (C) and IL-8 (D) mRNA expression in the presence of 100 nM DEX and MPA, and 1 µM TDF or DPV or combinations thereof. EtOH+DMSO (0.1% *v/v* for each) was used as the vehicle control. RNA was isolated, and cDNA was synthesized. Thereafter mRNA expression levels were determined by qRT-PCR and normalized to GAPDH mRNA expression levels. Relative fold change in expression was determined by setting vehicle control to 1. Pooled results of three or more independent experiments are shown and are represented as mean ± SEM. Statistical significance was assessed using a two-way ANOVA (A-B) with Tukey's multiple comparison post-test between the curves, although no statistical significance was obtained. Statistical significance was also assessed for C-D with a non-parametric Kruskal-Wallis test with a Dunn's multiple comparison post-test between the conditions. Statistical significance is denoted by *, ** and *** to indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Figure 3. Immunomodulatory effects of ARVs in PBMCs. Non-activated PBMCs were stimulated with TDF and DPV at 1 µM in the absence and presence of 100 nM DEX, or with the vehicle control (ETOH+DMSO, 0.1% *v/v* for each) for 48 h. RNA was isolated from PBMCs and thereafter cDNA was synthesized. Relative changes in GILZ (A), IL-6 (B), IL-8 (C) mRNA expression levels were determined by qRT-PCR and normalized to GAPDH mRNA expression levels. Relative fold change in expression was determined by setting vehicle control to 1. Pooled results of thirteen patients are shown with each condition done in triplicate and are represented as mean ± SEM. A Kruskal-Wallis test was performed with a Dunn's multiple comparison post-test to determine significant differences between treatments. Statistical significance is shown with * and **** denoting $p < 0.05$ and $p < 0.0001$.

948 **Figure 4. Immunomodulatory effects of ARVs in ectocervical explant tissue.** Ectocervical
949 explants were stimulated with 1 µM DPV or 100 nM DEX or MPA, or combinations thereof
950 as indicated. RNA was isolated and thereafter cDNA was synthesized. Relative changes in
951 GILZ (A), IL-6 (B), IL-8 (C) mRNA expression levels were determined by qRT-PCR and
952 normalized to GAPDH mRNA expression levels. Relative fold change in expression was
953 determined by setting vehicle control to 1. Pooled results of matched incubations performed
954 on seven donors are shown with each condition done in triplicate and are represented as mean
955 ± SEM. A Kruskal-Wallis test was performed with a Dunn's multiple comparison post-test to
956 determine significant differences between treatments. Statistical significance is shown with *
957 and ** denoting $p < 0.05$ and $p < 0.01$.

958 **Figure 5: Effect of TDF and DPV on the transcriptional efficacy and potency of GR,**
959 **PR-B or AR in the presence of their agonists.** U2OS cells were seeded into 10 cm^2 plates at
960 a density of 1.5×10^5 and incubated for 24 h. Thereafter, cells were transiently transfected for
961 24 h with pTAT-GRE-LUC, pcDNA-3 (empty vector) or the receptor expression vectors,
962 pSV-hAR, pcDNA3-hGR-WT or pSG5-PRB. Cells were re-seeded into 96-well plates at a
963 density of 1×10^4 cells/well for GR and AR, and 5×10^4 cells/well for PR-B. The cells were
964 then treated with increasing concentrations of the receptor agonists MIB, DEX and LNG in
965 the absence or presence of 1 µM TDF and DPV. EtOH+DMSO (0.1% *v/v* for each) was used
966 as the vehicle control and cells were incubated for 24 h. Cells were lysed and the luciferase
967 activity was measured for GR (A-B), AR (C-D), and PR-B (E-F). Luciferase activity was
968 normalized to protein content per well as determined by Bradford assay. Furthermore,
969 luciferase activity was normalized to the plateau value of the reference ligand (DEX, MIB,
970 and LNG) which was set to 100% to obtain relative fold induction. Pooled results from three
971 or more independent experiments are shown and data are represented as mean ± SEM.
972 Unpaired *t*-tests were used to obtain statistical significance of efficacies and potencies.

Figure 6: Dose-dependent effects of TDF on ligand-independent PR-B activation. U2OS cells were seeded and incubated for 24 h. Thereafter, cells were transiently transfected for 24 h with pTAT-GRE-LUC, pcDNA-3 (empty vector) or pSG5-PRB. Cells were then re-seeded and treated with varying concentrations of TDF (10^{-9} M - 10^{-6} M) in the absence and presence of 100 nM P4 for 24 h. Cells were lysed and luciferase activity was measured. Luciferase activity was normalized to protein content per well as determined by Bradford assay and to vehicle control (0.1% EtOH, 0.1% DMSO), to obtain normalized relative fold induction. Pooled results from three independent experiments are shown and data are represented as mean ± SEM. Unpaired *t*-tests were used to obtain statistical significance of efficacies and potencies.

Figure 7: Effect of ARVs on endogenous PR activity in the presence of LNG in MDA-MB-231 cells. PR-B stably-transfected MDA-MB-231 cells were transiently transfected with 9 µg pTAT-GRE-LUC and thereafter re-seeded into 96 well plates at a density of 1×10^4 cells/well. Cells were treated with increasing concentrations of LNG in the absence and presence of 1 µM TDF and DPV for 24 h. EtOH+DMSO (0.1% *v/v* for each) was used as the vehicle control and cells were incubated for 24 h. Cells were lysed and the luciferase activity was measured for PR. Luciferase activity was normalized to protein content per well as determined by Bradford assay. Furthermore, luciferase activity was normalized to the plateau of the reference ligand LNG which was set to 100% to obtain relative fold induction. Pooled results from four independent experiments are shown and data are represented as mean ± SEM. Unpaired *t*-tests were used to obtain statistical significance of efficacies and potencies.

Figure 8. Inhibition of HIV-1 infection by TDF and DPV in the absence and presence of steroid receptor agonists. TZM-bl cells were exposed to increasing concentrations of TDF or DPV in the absence and presence of 100 nM DEX and 0.01 nM MIB (A-B), or 100 nM

MPA and LNG (C-D) for 24 h prior to exposure to HIV-$1_{BaL\text{-}Renilla}$ in the presence of the compounds for 48 h. EtOH+DMSO (0.1% *v/v* for each) was used as the vehicle control. HIV-1 infection was determined by measuring HIV-1 long terminal repeat (LTR) activity by relative luciferase units (RLU) using BrightGlo (Promega), and these were normalized to the corresponding MTT absorbance readings (RLU/MTT). Pooled results from four independent experiments are shown where each condition was tested in triplicate and data are represented as mean ± SEM.

Tables

Table 1: Intravaginal and plasma or serum concentrations of ARV and progestin candidates for MPTs

	Product	Concentration released in product	Cervicovaginal fluid concentration (µM)	Cervical tissue concentration (µM)	Plasma or serum concentrations (nM)	References
ARVs	TFV gel	1% (*v/v*) gel*	41 – 34 000	0.7 – 4 800	11.83^{+}	(79, 80)[1,1]
	TDF ring	365 mg[#]	88 – 236	9 – 30	1.2 – 5.2	(5)[1]
	TDF (HAART)	300 mg	n.d	n.d	1100 - 1140	(81)[1]
	TDF/ FTC oral PrEP	300 mg TDF 200 mg FTC	n.d	79^{+}	77 - 87	(82)[2]

	DPV ring	25 mg[#]	17[+]	2 – 21	0.7 - 0.89	(7, 9, 54)[1,1,2]
Progestins	DMPA-IM (injectable)	150mg	n.d	n.d	21[+]	(70, 83, 84)
	ETG/EE ring (NuvaRing®)	120 µg ETG** 15 µg EE	n.d	0.002[+] ETG 0.0004 EE	3.7[+] ETG 0.05[+] EE	(85)[2]
	LNG-IUD (Mirena®)	30 µg** 52 mg	100 - 370	2.5[+]	0.2[+] 0.97[+]	(48, 86)[1, 1]
	LNG (implant, oral pill)	150 mg (implant) 1.5 mg (oral pill)	n.d	n.d	0.3 - 28	(87-89)[2, 2 , 2]
	NES/EE ring	150 µg NES** 15 µg EE	n.d	n.d	0.3[+]	(90)[2]
MPT	DPV+LNG ring	200 mg DPV[#] 320 mg LNG	n.d	n.d	2.1[+] DPV 5.1[+] LNG	(91)[1]
	TFV+LNG ring	8 – 10 mg TFV** 20 µg LNG	34 – 3484 TFV LNG - n.d	6.9 – 2865 TFV LNG - n.d	8[+] TFV 0.9 – 1.6 LNG	(92)[1]

1014 Notes: * - pericoitally applied, [#] - single dose, ** - daily dose, [+] - mean concentration, n.d – not determined, 1 –

1015 plasma concentrations, 2 – serum concentrations.

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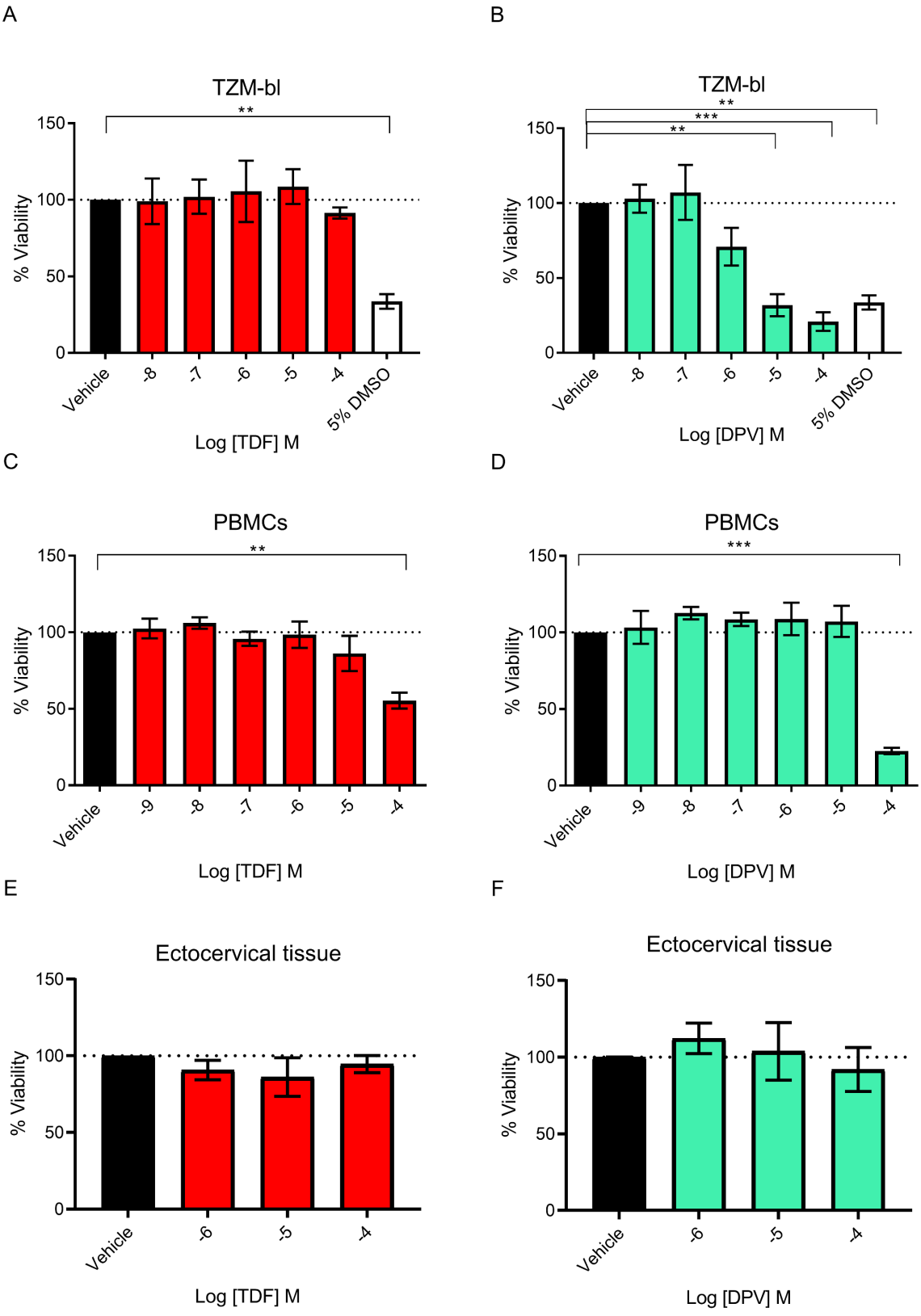
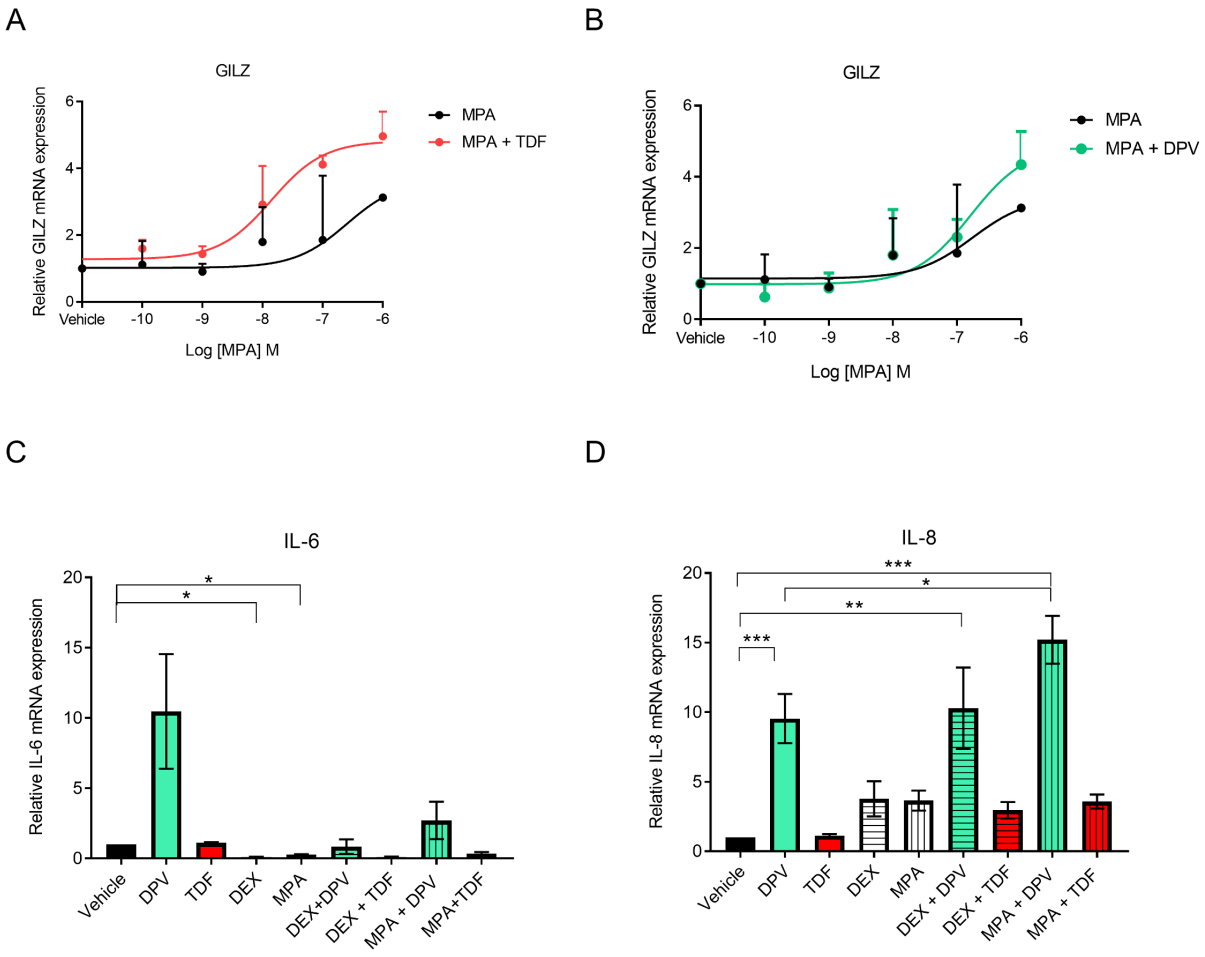


Figure 1



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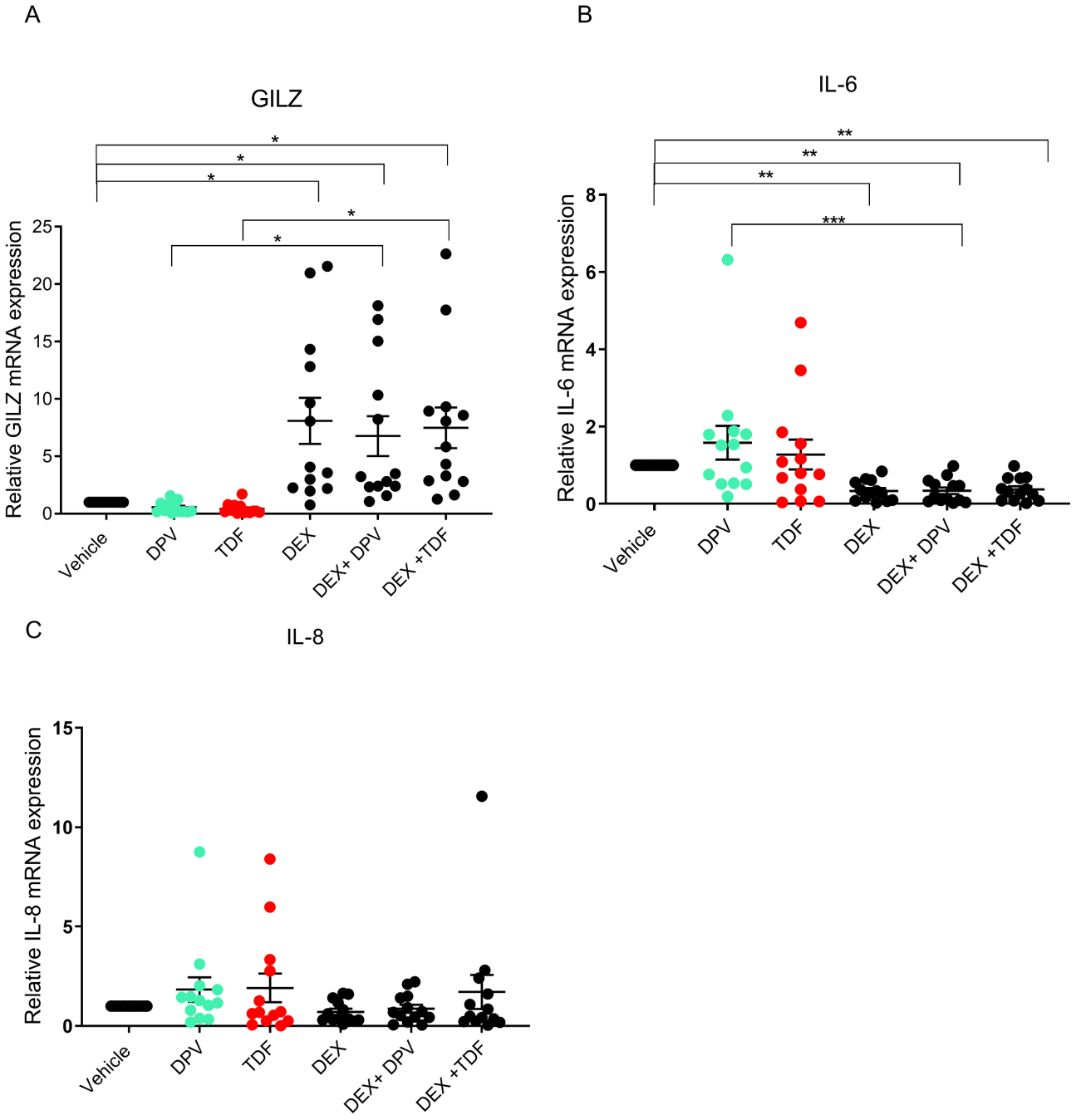

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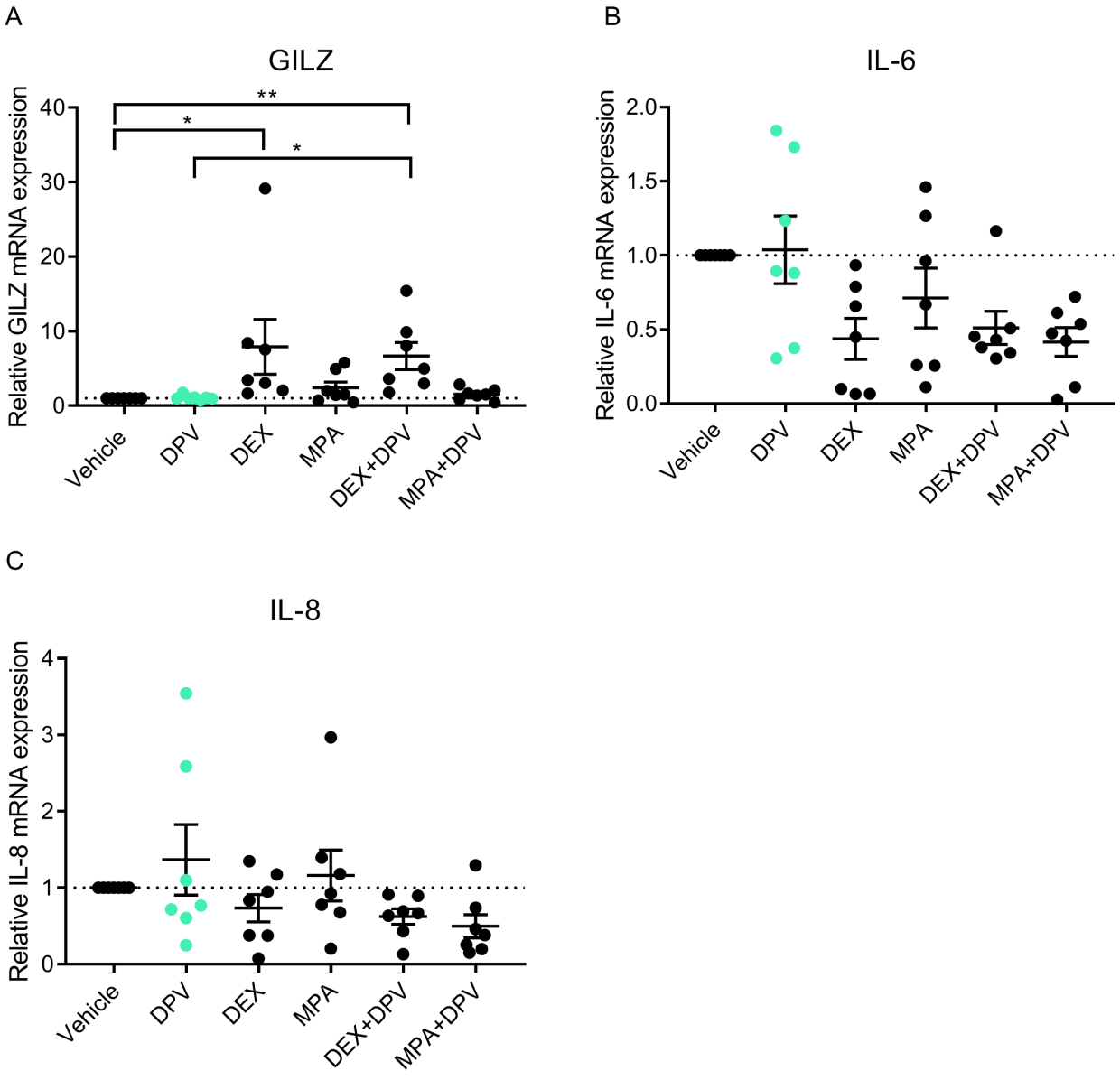
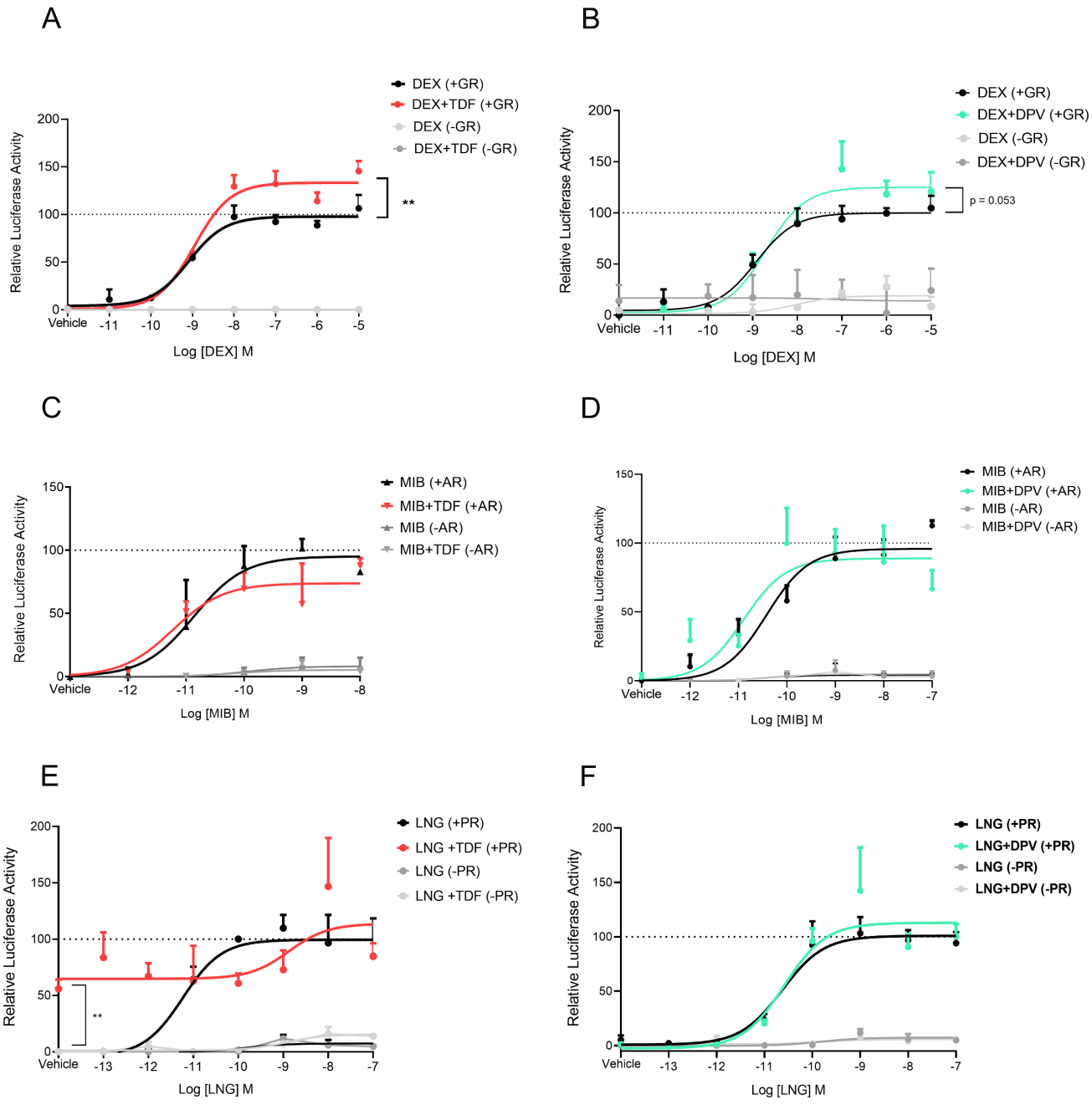


Figure 4

Antimicrobial Agents and Chemotherapy



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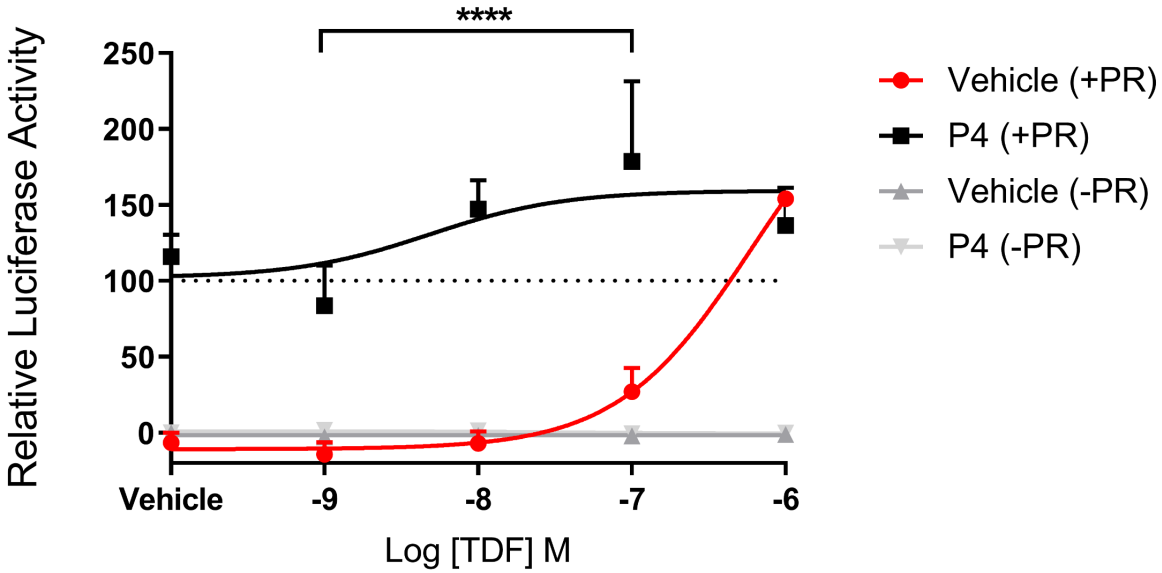

Figure 6

Antimicrobial Agents and Chemotherapy

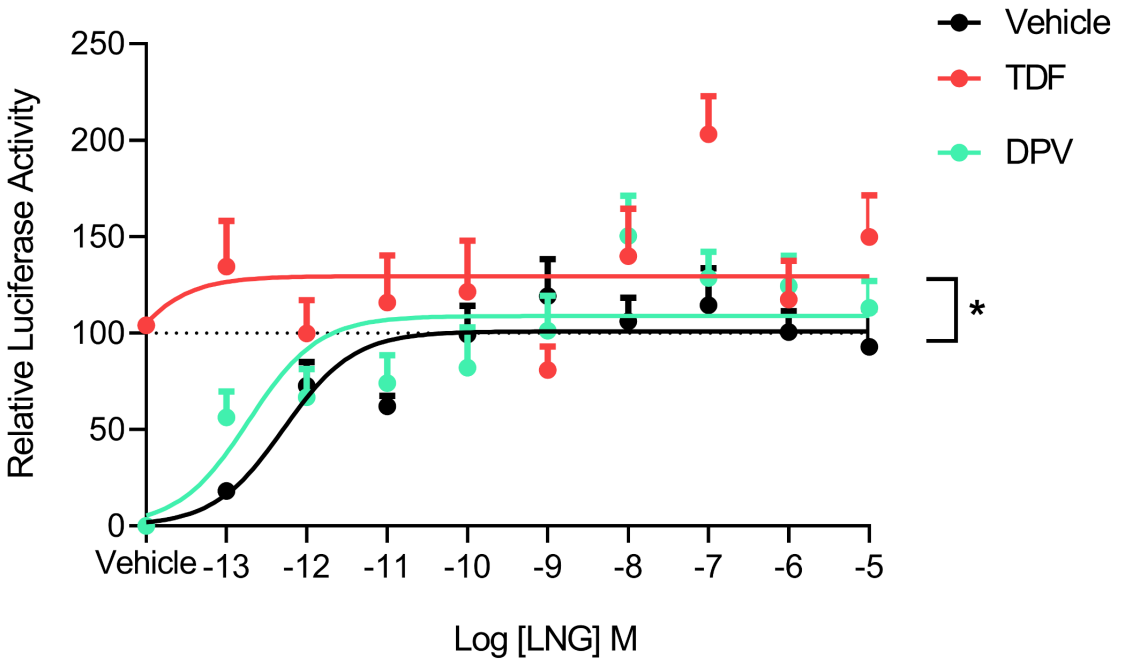

Figure 7

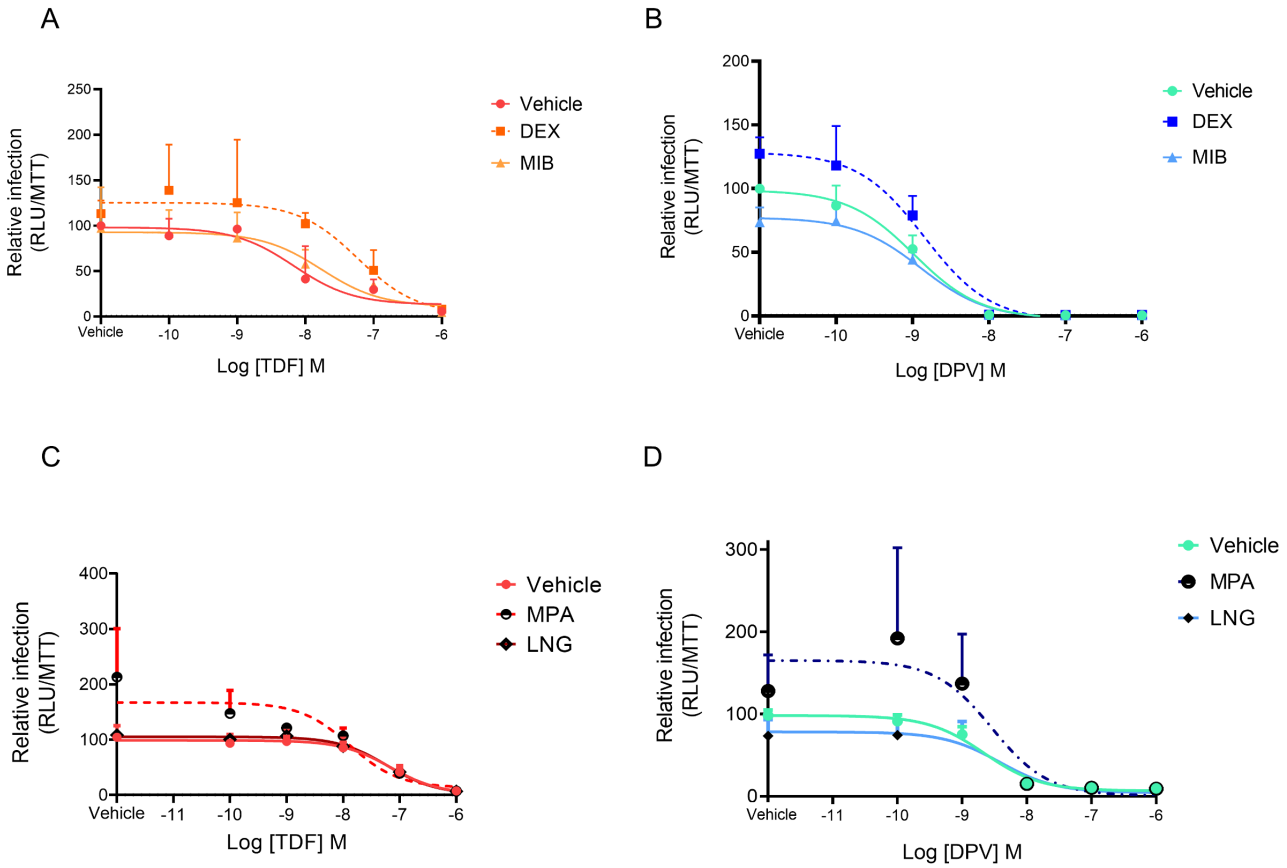

Figure 8

Antimicrobial Agents and Chemotherapy