In vivo modulation of cervicovaginal drug transporters and tissue distribution by film-released tenofovir and darunavir for topical prevention of HIV-1

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ABSTRACT

Clinical trials have demonstrated partial protection against HIV-1 infection by vaginal microbicide formulations based on antiretroviral (ARV) drugs. Improved formulations that will maintain sustained drug concentrations at viral target sites in the cervicovaginal mucosa are needed. We have previously demonstrated that treatment of cervicovaginal cell lines with ARV drugs can alter gene expression of drug transporters, suggesting that the mucosal disposition of ARV drugs delivered vaginally can be modulated by drug transporters.

This study aimed to investigate *in vivo* modulation of drug transporter expression in a non-human primate model by tenofovir and darunavir released from film formulations.

Cervicovaginal tissues were collected from drug-naïve macaques and from macaques vaginally treated with film formulations of tenofovir or darunavir. Drug release in vaginal fluid as well as drug absorption in cervicovaginal tissues and lymph nodes were verified by mass spectrometry. The effects of exposure to drugs on the expression of transporters relevant to ARV drugs were evaluated by quantitative PCR.

We showed expression in cervicovaginal tissue of drug-naïve macaques of transporters important for distribution of ARV drugs, albeit at lower levels compared to human tissue for key transporters including P-glycoprotein. Concentrations of tenofovir and darunavir well above the EC_{50} values determined *in vitro* were detected in vaginal fluid and vaginal tissues of macaques treated with drug-dissolving films over 24 hours and were also comparable to those shown previously to modulate drug transporter expression. Accordingly, Multidrug Resistance associated Protein 2 (MRP2) in cervicovaginal tissue was upregulated by both tenofovir and darunavir. The two drugs also differentially induced and/or inhibited expression of key uptake transporters for reverse transcriptase inhibitors and protease inhibitors.

The lower expression of key transporters in macaques may result in increased retention of ARV drugs at the simian cervicovaginal mucosa compared to the human mucosa and has implications for translation of pre-clinical data. Modulation of drug transporter expression by tenofovir and darunavir points to the potential benefit of MRP2 inhibition to increase ARV drug penetration through the cervicovaginal epithelium.

Key Words: Multidrug resistance associated protein, tenofovir, darunavir, microbicides, vagina, macaques

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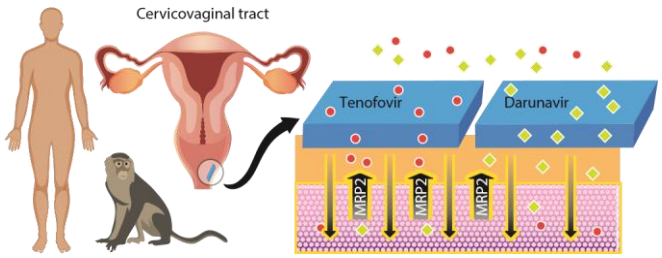

INTRODUCTION

Topical administration of antiretroviral (ARV) drugs to HIV-uninfected individuals who are at risk of becoming infected (pre-exposure prophylaxis, PrEP) has had variable outcomes. The promising results of the CAPRISA 004 trial (1) of tenofovir formulated as a vaginal gel were not confirmed in two subsequent clinical trials (2, 3) with the lack of protection being attributed primarily to low adherence (2-4). In contrast, two phase III trials of sustained release vaginal ring formulations of the non-nucleoside reverse transcriptase inhibitor, dapivirine, showed significant protection (37% and 31%) (5, 6). Sub-group analyses indicated higher protection (56%) in women aged >21 confirming the positive correlation of protection with adherence (6).

There has been much speculation as to the reasons for the inability to achieve higher protection. Uptake and persistence of topically applied ARV drugs may be influenced by membrane-bound ATP-binding cassette (ABC) solute efflux transporters and solute carrier (SLC) uptake transporters (7, 8). We, and other groups, recently demonstrated expression of ABC and SLC transporters involved in transport of ARV drugs in human cervicovaginal tissues (9-12) and colorectal tissue (13-16) . We demonstrated expression of efflux transporters both in colorectal epithelial cells (13) and submucosal lymphocytes (14) suggesting that drug transporters could alter the disposition of topically-applied ARV drugs at the epithelial barrier and in sub-epithelial CD4+ T cells. That efflux transporters can alter tissue levels of ARV drugs *in vivo* has been demonstrated in a murine model of vaginal administration of tenofovir where inhibition of the efflux transporter ABCC4/MRP4 resulted in significantly higher concentrations of drug in cervical and vaginal tissues (17).

In addition to being substrates for a number of ABC and SLC transporters, ARV drugs themselves are able to induce and/or inhibit expression and activity of drug transporters (8, 18). This raises the possibility of drug-drug interactions at mucosal tissues in the context of development of microbicides based on combinations of ARV drugs (19-22). Intestinal expression of efflux transporters is significantly altered in patients receiving ARV therapy compared to therapy-naive HIV-infected individuals (23). We previously investigated the effect of tenofovir and darunavir on expression of drug transporters in human epithelial cell lines. Stimulation of a panel of vaginal cell lines with soluble darunavir consistently increased expression of MRP efflux transporters (11) with the potential to influence uptake

of several protease inhibitors (PIs) and nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) including tenofovir (24, 25) .

Nonetheless, expression of drug transporters in cell lines does not fully reflect expression in equivalent *ex vivo* tissue samples (11). In addition, expression analyses *in vitro* do not mimic closely the complexity of the physiological environment of the cervicovaginal tract where expression of drug transporters may be influenced by pH variations, hormones, mucus, microbiota, anti- and pro- inflammatory mediators. For the first time, to the best of our knowledge, we provide a comprehensive description of drug transporter expression in the female genital tract of the cynomolgus macaque - a common non-human primate model for HIV transmission (26-29).

We also describe *in vivo* modulation of expression of drug transporters following vaginal administration of tenofovir or darunavir. For these experiments, we used film formulations of both drugs. In a human study, vaginal delivery of the non-nucleoside RT inhibitor dapivirine was shown to be equally effective in film or gel formulations (30). Films may be a more effective dosage form since there is no requirement for an applicator and no product leakage due to the reduced volume of material applied and lack of liquid component in the formulation (31).

EXPERIMENTAL SECTION

Ethics statement

Non-human primates were used in accordance with French national regulations and under the supervision of national veterinary inspectors (CEA Permit Number C 92-032-02). All experiments were carried out in conformity with European Directive 2010/63/EU and the Weatherall Report. The CEA complies with the Standards for Humane Care and Use of Laboratory Animals of the Office for Laboratory Animal Welfare (OLAW, USA). The study was approved by “Comité Régional d'Ethique pour l'Expérimentation Animale Ile-De-France Sud” (statement number 14-041). Ethical approval was obtained for testing of the tenofovir and darunavir formulations, but not for the respective placebo formulations.

Study setting and animal housing

Animal experiments were conducted at “Commissariat à l’Energie Atomique”, IDMIT infrastructure, Fontenay-aux-Roses. Subsequent gene expression and mass spectrometry analyses of macaque samples were carried out jointly at the University of Aberdeen and University of Siena, and King’s College London respectively.

Adult female cynomolgus macaques (*Macaca fascicularis*, weight range 3–5 kg) of Mauritian origin were included in the study. Animal routine heath monitoring was carried out twice daily and supervised by the veterinarians in charge of the CEA animal facility. Animals were fed twice daily with commercial monkey chow and fresh fruits by trained personnel. Macaques had constant access to water supply. Macaques were provided with environmental enrichment including toys, novel foodstuffs, music and regular interaction with caregivers and research staff supervised by the CEA Animal Welfare Body. Experimental procedures were conducted after animal sedation with 10 mg/kg (body weight) of ketamine chlorhydrate.

Tenofovir and darunavir film formulations

Darunavir and tenofovir dissolving films were manufactured as previously described (13). For preparation of darunavir-based dissolving films micronized darunavir was added to a mixture of tween 80 (0.55%) in water and homogenized. PEO-10 (1.10 %) and PEO WSR301 (0.055 %) were then added followed by PEG 1000 (0.916 %), and finally, once homogenised, HPMC E50 (5.310 %). For preparation of tenofovir-based dissolving films, tenofovir was homogenized with a mixture of glycerine (3.33 %) and water. NaOH at 1 M was added until tenofovir was completely dissolved. A premixed solution of propyl paraben (0.01 %)/PECOL (0.83 %) and alcohol (28.07 %) was added to the tenofovir solution followed by addition of PEO 205 (1.00 %) and Benecel E50 (5.00 %) sequentially. Both darunavir and tenofovir dispersions were bath-sonicated to remove air, coated onto release paper using a Coatema Easycoater, set for 1400 µm wet thickness, and dried at 37°C.

Dissolution of tenofovir and darunavir film formulations was tested by incubation of single films in 25mM ammonium acetate, pH 4.2, 500ml. Tenofovir film dissolved completely after 2 hours while darunavir dissolution was slower (4 hours) and more variable.

Administration of tenofovir and darunavir dissolving films and sample collection in macaques

Films containing 7 mg tenofovir (7.9% drug loading) or 1.7 mg darunavir (6.2% drug loading) were atraumatically inserted into the vagina of female cynomolgus macaques on day 0 (n=4 for each drug). For this procedure, animals were sedated with ketamine chlorhydrate, and placed in a ventral recumbency position with hips elevated. Rolled film was inserted atraumatically into the vaginal vault near to the cervix. Vaginal fluids were collected using Weck-Cel® spears (Beaver Visitec International) at 0, 1, 2, 4, 8, 24, 48, 72 hours and 7 days after administration of the film, as described previously (22). Pre-weighed Weck-Cel spears were placed in the vaginal vault for 1-2 min to absorb fluid. Upon removal, sponges were reweighed to calculate the collected vaginal fluid weight and stored at –80°C until drug quantitation analyses by HPLC-MS/MS.

After collection of vaginal fluid on day 7, a vaginal wash was administered to all animals. Following a 3-week wash-out period, tenofovir and darunavir films were again administered with each macaque receiving an identical film to that used previously. Animals were necropsied 1 hour (two animals per film) or 24 hours (two animals per film) after vaginal administration of the tenofovir or darunavir film formulation and cervicovaginal and colorectal tissues as well as lymph nodes were sampled and stored at -80°C for subsequent determination of drug levels in tissues and drug transporter (cervicovaginal tissue only). For baseline drug transporter expression analyses vaginal and cervical tissues were collected from seven necropsied drug-naïve cynomolgus macaques. Samples were stored at-80°C until RNA was isolated.

Quantification of drugs in tissues by mass spectrometry

Tenofovir and darunavir in Weck-Cel spears were determined by HPLC-MS/MS using the Thermo Scientific Accela Pump and Autosampler coupled to a Thermo Scientific LTQ XL mass spectrometer. Adefovir (for tenofovir) and darunavir-^{13}C6 were used as internal standards.

Weck-Cel spears were extracted with 20% methanol solution or acetonitrile for tenofovir or darunavir, respectively. To confirm quantitative extraction, blank Weck-Cel spears were spiked with standard amounts of drug and then extracted as above. Eluted drugs were compared with standard solutions. Both methanolic and acetonitrile extracts (50 µl) were combined with internal standard and diluted 20 times with 0.1% formic acid in 50% acetonitrile and transferred to injection vials. The calibration range for the vaginal fluid

assays, using a 10 µL injection volume, was 0.05–300 µg for both drugs. Both drugs were chromatographically separated from the respective internal standards using a reversed phase Thermo Hypersil Gold aQ, 150 x 2.1 mm, 3 µ column.

Tenofovir and Adefovir were separated with run time of 10 min. Initial conditions consisted of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at 95/5 (v/v) with a column temperature of 40 °C and a flow rate of 0.2 mL/min. The gradient conditions were: 0-1.0 min 5% B, 1.0-5.0 min 5-80% B, 5.0-6.0 min 80% B, 6.0-6.2 min 80-5% B, 6.2-10.0 min 5% B for re-equilibration. Under these conditions tenofovir and adefovir displayed retention times of approximately 2.2 and 2.1 min respectively.

Darunavir and darunavir-^{13}C6 were resolved with run time of 8 min. Initial conditions consisted of mobile phase A (5mM NH_4HCO_2 in water) and mobile phase B (5mM NH_4HCO_2 in 95% acetonitrile) at 30/70 (v/v) with a column temperature of 40 °C and a flow rate of 0.2 mL/min. The gradient conditions were: 0-0.2 min 70% B, 0.2-2.0 min 70-90% B, 2.0-4.0 min 90% B, 4.0-4.2 min 90-70% B, 4.2-8.0 min 70% B for re-equilibration. Under these conditions darunavir and darunavir-^{13}C6 displayed retention time of approximately 2.2 minutes.

The mass spectrometer was operated in positive ion electrospray full MS/MS scan mode. For tenofovir determination, ion source parameters were as follows: I spray Voltage 4 kV, Capillary Voltage 6.0 V, Capillary Temperature 350°C, Sheath Gas flow 50 Arb. and Auxiliary Gas flow 0 Arb. The analyte and its internal standard product ions were monitored using collision energy set at 30 Arb. for both tenofovir and adefovir. Full MS/MS mass ranges were m/z 288.3→174.0-210.0, and 274.3→190.0-230.0 for tenofovir and adefovir, respectively. For darunavir determination, ion source parameters were as follows: I spray Voltage 4 kV, Capillary Voltage 38.0 V, Capillary Temperature 400°C, Sheath Gas flow 50 Arb. and Auxiliary Gas flow 10 Arb. The analyte and its internal standard product ions were monitored using collision energy set at 30 Arb. for both darunavir and darunavir-^{13}C6. Full MS/MS mass range were m/z 548.2→350.0-450.0, and 554.2→350.0-450.0 for darunavir and darunavir-^{13}C6, respectively. Each transition was monitored with a dwell time of 0.25 s.

Tenofovir and darunavir in tissues (vaginal, cervical, uterine and rectal) and lymph nodes were determined by HPLC-MS/MS using the Thermo Scientific Vanquish pump and autosampler coupled to a TSQ Vantage mass spectrometer. Tissue samples were

homogenised in acetonitrile/0.1% formic acid for 20 min at 30Hz using the TissueLyser II (Qiagen) and centrifuged (20,000 x g, 10 min). Supernatants were combined with internal standard, diluted with 0.1% formic acid in 70% /50% acetonitrile and transferred to injection vials. For higher tissue concentrations, the calibration range was 0.05 -25 µg/ml for both drugs. For lower tissue concentrations, calibration ranges were 0.1-50 ng/ml (tenofovir) and 0.1-20ng/ml (darunavir). Samples were resolved by reversed-phase HPLC using the Hypersil Gold aQ column (as above) for darunavir with identical gradient and internal standard. For tenofovir, the Zorbax Eclipse Plus (100 x 2.1mm, 1.8µ) column (Agilent Technologies) was used with identical mobile phase buffers and flow rates but a modified gradient. Run time was 8 min: 0-1.0 min 5% B, 1.0-2.5 min 5%-25% B, 2.5 – 3.0 min 25% -80% B, 3.0 – 3.5 min 80% B, 3.5 - 4.0 min 80 - 5% B, 4.0 - 8.0 min 5% B. Retention times were 1.3 min and 1.2 min for tenofovir and adenovir, respectively.

The mass spectrometer was operated in positive ion electrospray full MS/MS scan mode. For tenofovir determination, ion source parameters were as follows: Spray Voltage 3.5 kV, S-Lens RF Amplitude 128 V, Capillary Temperature 350°C, Vaporizer Temperature 350°C, Sheath Gas flow 60 Arb. and Auxiliary Gas flow 0 Arb. The analyte and its internal standard product ions were monitored using collision energy set at 25 and 30 Arb. respectively. MRM transitions were m/z 288.05→159.10, 288.05→176.10, 288.05→206.10 for tenofovir, and 274.03→162.10 for adefovir.

For darunavir determination, ion source parameters were as follows: Spray Voltage 3 kV, S-Lens RF Amplitude 114 V, Capillary Temperature 360°C, Vaporizer Temperature 350°C , Sheath Gas flow 50 Arb. and Auxiliary Gas flow 10 Arb. The analyte and its internal standard product ions were monitored using collision energy set at 15 Arb. for both darunavir and darunavir-^{13}C6. MRM transitions were m/z 548.13→392.25, 548.13→436.25 for darunavir, and 554.14→398.30 for darunavir-^{13}C6.

Cell lines

The VK2/E6E7, HEC-1A, End1/E6E7 and Ect1/E6E7 human cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown as previously reported (11). Cells were stimulated with the highest non-cytotoxic concentrations of darunavir (250 µM) and tenofovir (1mM) as determined by the TACS-XTT Cell Proliferation Assay Kit (Trevigen, Gaithersburg, USA). To obtain these concentrations the drug dissolving

films and the respective placebo formulations (1.5 cm^2) were cut in three (darunavir) and six (tenofovir) sections of equal size using a sterile scalpel. Individual film portions were added to the cell culture medium and cells were incubated at 37°C with 5% CO_2 for 72 hours. After incubation cells were harvested for total RNA extraction. Five biological replicates for all experimental conditions were carried out.

RNA isolation and quantitative PCR

Vaginal and cervical tissues (three samples each per animal), from drug-naïve and drug-exposed animals, were homogenized in QIAzol Lysis Reagent using 5mm stainless steel beads in Tissue Lyser (all from Qiagen, Stockach, Germany). Total RNA was extracted with the RNeasy Plus Universal Mini Kit (Qiagen) following the manufacturer's protocol. RNA was extracted from cell lines using the NucleoSpin RNA II Isolation Kit (Macherey-Nagel, Germany) as previously described (11). Following DNase treatment on column, integrity and quantification of RNA were evaluated on the Agilent 2100 Bioanalyzer (Agilent Technologies). Samples with RNA integrity values (RIN) >6 were included in the analyses. Samples were stored at −80ºC. Reverse transcription and quantitative PCR were performed with a previously described method (11) using custom-made primers for *Macaca fascicularis* genes where required. Initial screening of expression of ARV drug transporters was performed using the TaqMan® PCR Array Human Drug Transporters Fast 96-well system and the Fast Advanced Master Mix (Life Technologies Italia, Monza, Italy). This PCR array is pre-configured with lyophilised TaqMan probes and primers directed to 84 human drug transporter genes most of which were compatible with the orthologous *M. fascicularis* genes. For the endogenous control gene HPRT1 and genes encoding BCRP, MRP3, ABCD3, OAT3 and CNT2 transporters, Ct values were measured using custom-made Taqman® assays for *M. fascicularis* genes (Life Technologies). DNA amplifications (in triplicate) were performed with the Viia7 Real-time PCR cycler (Life Technologies) as previously described (11). Transcript levels of drug-naïve macaque tissue were calculated as ratio between the mean Ct value of the most stable endogenous control genes (HPRT1, RPLP0, UBC), determined on the basis of the lowest SD score (32), and the Ct value for the target gene. Expression analyses of tissue from drug-treated animals was targeted to the 20 genes (9 ABC transporters, 11 SLC transporters) that were shown to be expressed in drug-naïve macaques. Relative transcripts levels in tissue from drug-treated animals versus non-treated animals were determined using the comparative Ct method (33) .

Statistical analysis

All data are reported as the sample mean ± standard deviation (SD). Pairwise comparisons between means of different groups were performed using a Student *t*-test. To assess the difference between drug- and placebo-treated versus untreated cells, statistical analysis were performed by one-way ANOVA with Dunnett's post hoc test. Significance was set at $P<0.05$. Analysis were conducted using the GraphPad Prism software version 6 (La Jolla, CA).

RESULTS

Comparative analyses of expression of drug transporters in the cervix and vagina of non-treated macaques and humans.

To detect efflux and uptake transporters that may influence ARV permeability we screened expression of 84 drug transporter genes in non-treated macaques. Both cervical and vaginal macaque tissue expressed the twenty transporters for which ARVs are substrates and were identified previously in the human cervix (Table 1). However, some MRP, CNT and SLCO transporters that were expressed in macaque cervicovaginal tissue were not previously detected in human vagina (Table 1). Expression differences observed between cervical and vaginal macaque tissue were not significant. OAT transporters and OCT2, not expressed in cervicovaginal human tissue, were not detected in macaque tissue (data not shown). P-gp ($P<0.0001$), MRP7 ($P=0.0049$), MRP5 ($P<0.0001$), OCT3 ($P<0.0001$) and OATPB ($P=0.0018$) were expressed at significantly lower levels in macaque cervix and vagina when compared to human tissue from the equivalent anatomical sites (Table 1). MRP1 and OATPE expression was also significantly lower in macaque vaginal tissue compared with human ($P<0.0001$). Macaque cervical tissue showed significantly lower expression of MRP3, OCT1 (both $P<0.0001$), CNT3 ($P=0.048$) and OATP8 ($P<0.0001$) (Table 1) compared to human tissue. Expression levels of the other simian genes shown in Table 1 were not significantly different from the human homologues.

Table 1. Drug transporter gene expression in macaque and human cervicovaginal tissue.

Gene	Gene expression level †				
	Macaque tissues		Human tissues‡		
	Cervix	Vagina	Ectocervix	Endocervix	Vagina
P-gp	0.65 (±0.05)	0.61 (±0.02)	0.87 (±0.05)	0.79 (±0.02)	0.85 (±0.04)
MRP1	0.77 (±0.04)	0.75 (±0.02)	0.86 (±0.02)	0.76 (±0.03)	0.84 (±0.00)
MRP7	0.71 (±0.02)	0.70 (±0.02)	0.81 (±0.03)	0.75 (±0.03)	0.82 (±0.04)
MRP2	0.64 (±0.02)	0.61 (±0.01)	- §	0.60 (±0.10)	-
MRP3	0.60 (±0.03)	0.56 (±0.03)	0.81 (±0.05)	0.73 (±0.01)	-
MRP4	0.73 (±0.06)	0.67 (±0.02)	0.80 (±0.03)	0.74 (±0.04)	-
MRP5	0.70 (±0.02)	0.70 (±0.02)	0.92 (±0.03)	0.81 (±0.01)	0.91 (±0.04)
MRP6	0.60 (±0.05)	0.56 (±0.03)	0.66 (±0.03)	0.66 (±0.10)	-
BCRP	0.79 (±0.03)	0.78 (±0.04)	0.86 (±0.05)	0.79 (±0.03)	0.84 (±0.06)
OCT1	0.56 (±0.01)	0.54 (±0.03)	0.77 (±0.01)	0.66 (±0.11)	-
OCT3	0.59 (±0.01)	0.56 (±0.04)	0.88 (±0.06)	0.79 (±0.01)	0.89 (±0.08)
CNT1	0.66 (±0.07)	0.58 (±0.03)	0.77 (±0.01)	0.56 (±0.11)	-
CNT2	0.68 (±0.07)	0.57 (±0.06)	0.92 (±0.06)	0.64 (±0.16)	-
CNT3	0.71 (±0.07)	0.70 (±0.05)	0.83 (±0.09)	0.78 (±0.02)	-
OATPC	0.68 (±0.04)	0.64 (±0.05)	-	0.66 (±0.10)	-
OATP8	0.58 (±0.04)	0.55 (±0.04)	0.80 (±0.14)	0.77 (±0.03)	-
OATPD	0.71 (±0.10)	0.72 (±0.09)	0.81 (±0.01)	0.72 (±0.02)	0.82 (±0.01)
OATPE	0.68 (±0.06)	0.63 (±0.03)	0.81 (±0.02)	0.69 (±0.12)	0.83 (±0.00)
OATP	0.82 (±0.03)	0.73 (±0.09)	0.91 (±0.03)	0.83 (±0.04)	0.88 (±0.03)
OATPB	0.59 (±0.08)	0.59 (±0.08)	0.81 (±0.05)	0.74 (±0.02)	0.84 (±0.03)

† Values were calculated as Ct(control gene)/Ct(target gene). Results are reported as the mean (± standard deviation) of samples from each tissue. Statistically significant differential expression between macaque (n=7 vaginal, n=7 cervix) and human tissues (n=3 vaginal, n=4 endocervix, and n=4 ectocervix samples) is highlighted. Pairwise comparisons were macaque cervix vs human ectocervix + endocervix, macaque vagina vs human vagina. *P* values were as follows: P-gp, MRP1, MRP3, MRP5, OCT1, OCT3, OATP8, and OATPE ($P<0.0001$), OATPB ($P=0.0018$), CNT3 ($P=0.048$), MRP7 ($P=0.0049$). Significance was set at $P<0.05$.

‡Data are already reported in Hijazi *et al*. 2015.

§ Ct(target gene) > 35, not expressed.

Release and tissue distribution of darunavir and tenofovir *in vivo*

Release of drugs *in vivo*, following intravaginal administration of dissolving film formulations of darunavir or tenofovir to cynomolgous macaques was determined by measurement of levels in vaginal fluids. There were significant variations in drug concentrations between animals in the same group. Concentrations of darunavir reached a maximum of approximately 2.4 mM (T_{max} =2 hours) and decreased to approximately 1mM by 24 hours (Figure 1A). The higher dose of tenofovir in the film formulation resulted in C_{max} of approximately 20mM that may also reflect the higher solubility of the drug in aqueous solution. At 24 hours post administration, the concentration of tenofovir in vaginal fluid was also approximately 1mM (Figure 2A). Thus between 1 and 24 hours post administration of drugs, concentrations of darunavir and tenofovir were, respectively, more than 5 and 3 logs higher than the EC_{50} values determined *in vitro* (34, 35). These concentrations also exceed those shown to modulate expression of some drug transporters in previous *in vitro* studies (11). At 48 hours post administration the concentrations of tenofovir and darunavir in vaginal fluid remained above the EC_{50} values (approximately 426 µM and 144 µM, respectively).

To investigate tissue distribution of darunavir and tenofovir, the cynomolgus macaques were dosed with the same drug formulations as previously. Macaques (n=2 per drug per time point) underwent necropsy at 1 hour and 24 hours post drug administration and drug concentrations in vaginal (3 sites), cervical, uterine and rectal tissues were determined. Because there were relatively large variations (at most, 3 - 4 fold) in tissue concentrations of drug between animals at the same time point, the data are presented for individual animals (Figures 1B, 1C, 2B, 2C). At 1 hour, concentrations of darunavir in vaginal and cervical tissues were in the range of 15 -140µM. Lower concentrations of darunavir, that were nonetheless well above the EC_{50} value of 1-5nM (35), were measured in uterine (1.4, 2.5µM at 1h; 5.6, 1.3 µM at 24h) and rectal tissue (1.1, 0.27 µM at 1h; 1.8, 0.43 µM at 24h) (Figure 1B). In animals, necropsied at 24 hours, there were higher concentrations of darunavir in vaginal tissue which may indicate increased drug absorption (Figure 1C). Drug concentrations were also determined in iliac, inguinal and axillary lymph nodes ranging from 1.5 - 40 nM at 1 hour and 2.9 – 173 nM at 24 hours (Supporting Information, Supplemental figure 1). Concentrations of darunavir in vaginal tissue, but not in other tissues, were again comparable with those shown to modulate transporter expression (11).

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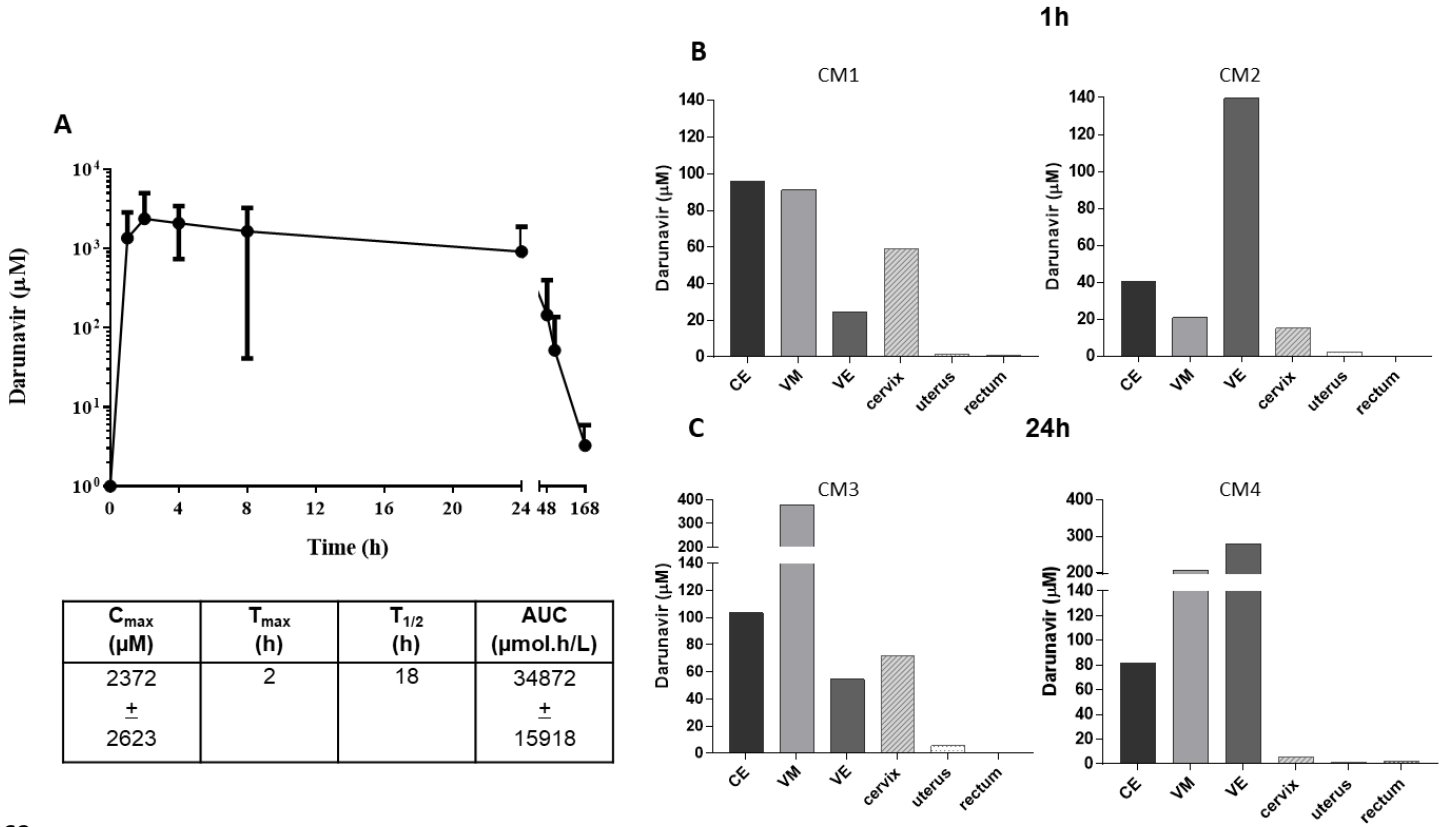
Figure 1. Darunavir release in cervicovaginal tissue and vaginal fluid. The dissolving film formulation of darunavir (1.7 mg) was administered vaginally to cynomolgous macaques (n=4). **A**) Darunavir concentrations in vaginal fluid collected at intervals over 24 hours (A). Pharmacokinetic parameters are shown below the line plot. **B-C**) Vaginal, cervical, uterine and rectal tissue were sampled post necropsy from 2 animals at 1 hour (CM1, CM2) and 24 hours (CM3, CM4). Concentrations of darunavir in fluid and tissue samples were determined by HPLC-MS/MS. CE: near the cervix; VM: middle of vaginal vault; VE: near to vaginal entrance; CM: cynomolgous macaque. Drug tissue concentrations are shown for individual animals as indicated.



C_{max} (µM)	T_{max} (h)	$T_{1/2}$ (h)	AUC (µmol.h/L)
2372 ± 2623	2	18	34872 ± 15918

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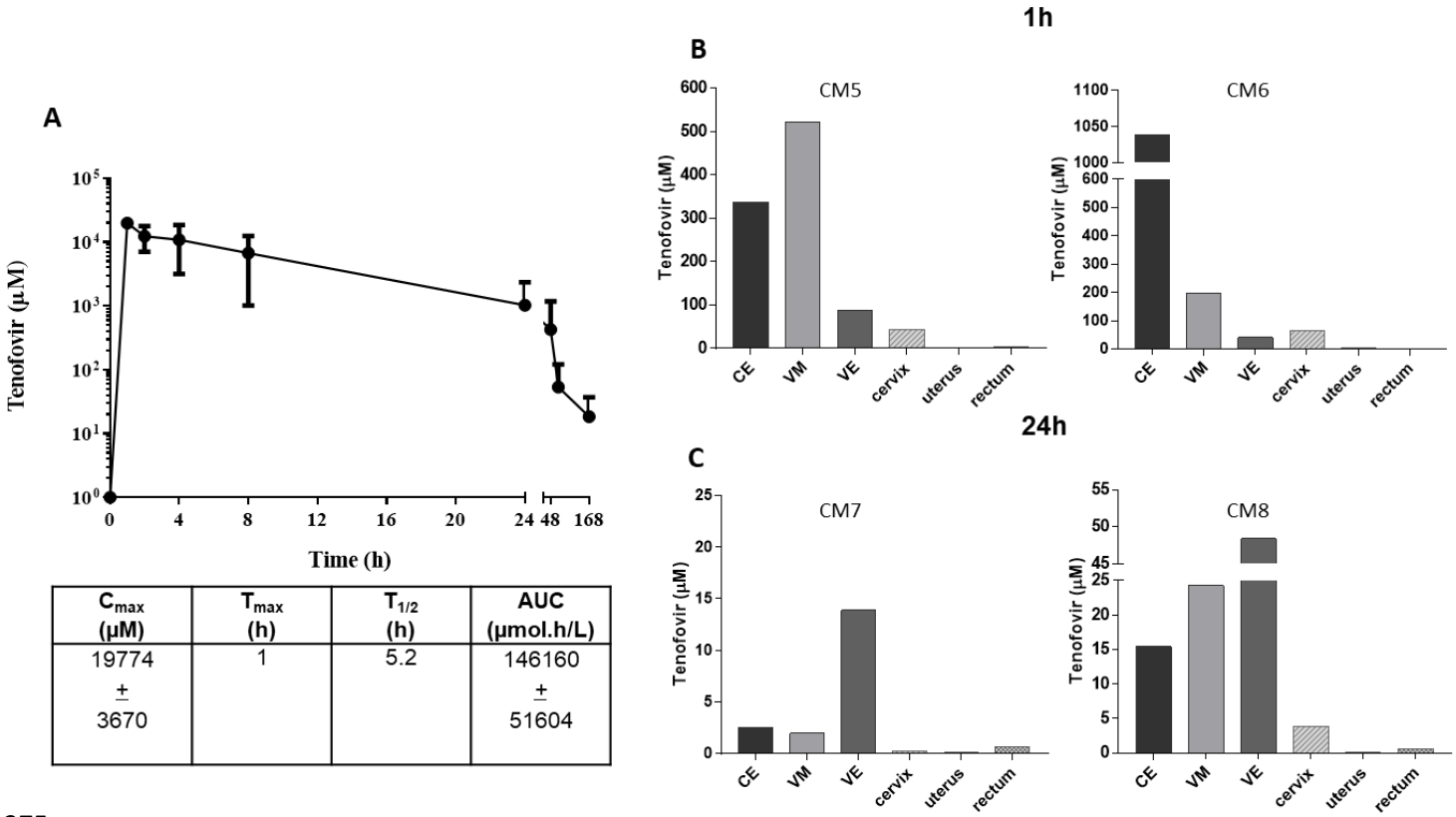
363

364 Concentrations of tenofovir in vaginal and cervical tissues at 1 hour post application were in
365 the range of 5 to 100-fold higher than the EC_{50} value while concentrations in uterine (2.4, 5.6
366 µM at 1 hour) and rectal tissue (2.7, 0.8 µM at 1 hour) were within or above the EC_{50} range
367 of 0.14 – 1.5 µM (34) (Fig. 2B). The concentrations in vaginal tissue were also comparable to
368 those shown previously to modulate drug transporter expression. Tenofovir concentrations in

lymph node tissues were well below EC_{50} values (Supporting Information, Supplemental figure 2). At 24 hours, all tissue concentrations were considerably reduced likely reflecting intracellular phosphorylation of tenofovir to tenofovir diphosphate, the active form of the drug (36).

Figure 2. Tenofovir release in cervicovaginal tissue and vaginal fluid. The dissolving film formulation of tenofovir (7 mg) was administered vaginally to cynomolgous macaques (n=4). **A**) Tenofovir measurements in vaginal fluid collected at intervals over 24 hours. Pharmacokinetic parameters are shown below the line plot. **B-C**) Vaginal, cervical, uterine and rectal tissue were sampled post necropsy from 2 animals at 1 hour (CM5, CM6) and 24 hours (CM7, CM8). Concentrations of tenofovir in fluid and tissue samples were determined by HPLC-MS/MS. CE: near the cervix; VM: middle of vaginal vault; VE: near to vaginal entrance; CM: cynomolgous macaque. Drug tissue concentrations are again shown for individual animals.



C_{max} (µM)	T_{max} (h)	$T_{1/2}$ (h)	AUC (µmol.h/L)
19774 ± 3670	1	5.2	146160 ± 51604

Effect of vaginal administration of film-released tenofovir or darunavir on expression of drug transporters.

Drug transporter gene expression in tissues collected from animals (n=2, for each drug) treated with film-formulations of tenofovir (7 mg) or darunavir (1.7 mg) was quantified relative to expression in non-treated animals. Tissues were collected at 24 hour post administration of drug. Drug concentrations in vaginal fluid taken at the same time point indicated similar levels of drug exposure in each pair: tenofovir concentrations were 0.7 and 1.4mM, respectively while darunavir concentrations were 1.8 and 2.2 mM. ABC and SLC transporters which were up- or down-regulated in the cervix and vagina after 24 hours by tenofovir are shown in Figure 3. Amongst efflux transporters MRP2 was significantly upregulated by tenofovir in both the cervix (7.5-fold, P=0.0082) and the vagina (4.2-fold, P=0.0047). Significant downregulation of MRP1 (0.18-fold; P=0.0159) and MRP7 (0.32-fold; P=0.02) by tenofovir was also evident in vaginal tissue. Amongst uptake transporters OATPE was significantly upregulated by tenofovir in both the cervix and the vagina (3.3 and 5.9-fold; P=0.016 and P=0.0001, respectively).

Figure 3. Drug transporter gene expression in tenofovir-treated macaques. Animals were vaginally treated with 7 mg tenofovir dissolving film. Levels of total RNA extracted from vaginal and cervical biopsies at 24 hours were measured by quantitative PCR. Relative quantitation was determined using the comparative Ct method with data normalized to three housekeeping genes (HPRT1, RPLP0, UBC) and calibrated to the average Ct of naïve animals (fold induction $2^{-\Delta\Delta Ct}$). Values of cervix **A**) and vagina **B**) are represented as mean ± SD of three samples carried out in triplicate on PCR runs. Statistical analysis was performed using *t*-Student's test (*$P\leq0.05$, **$P\leq0.01$ and *** $P\leq0.001$).

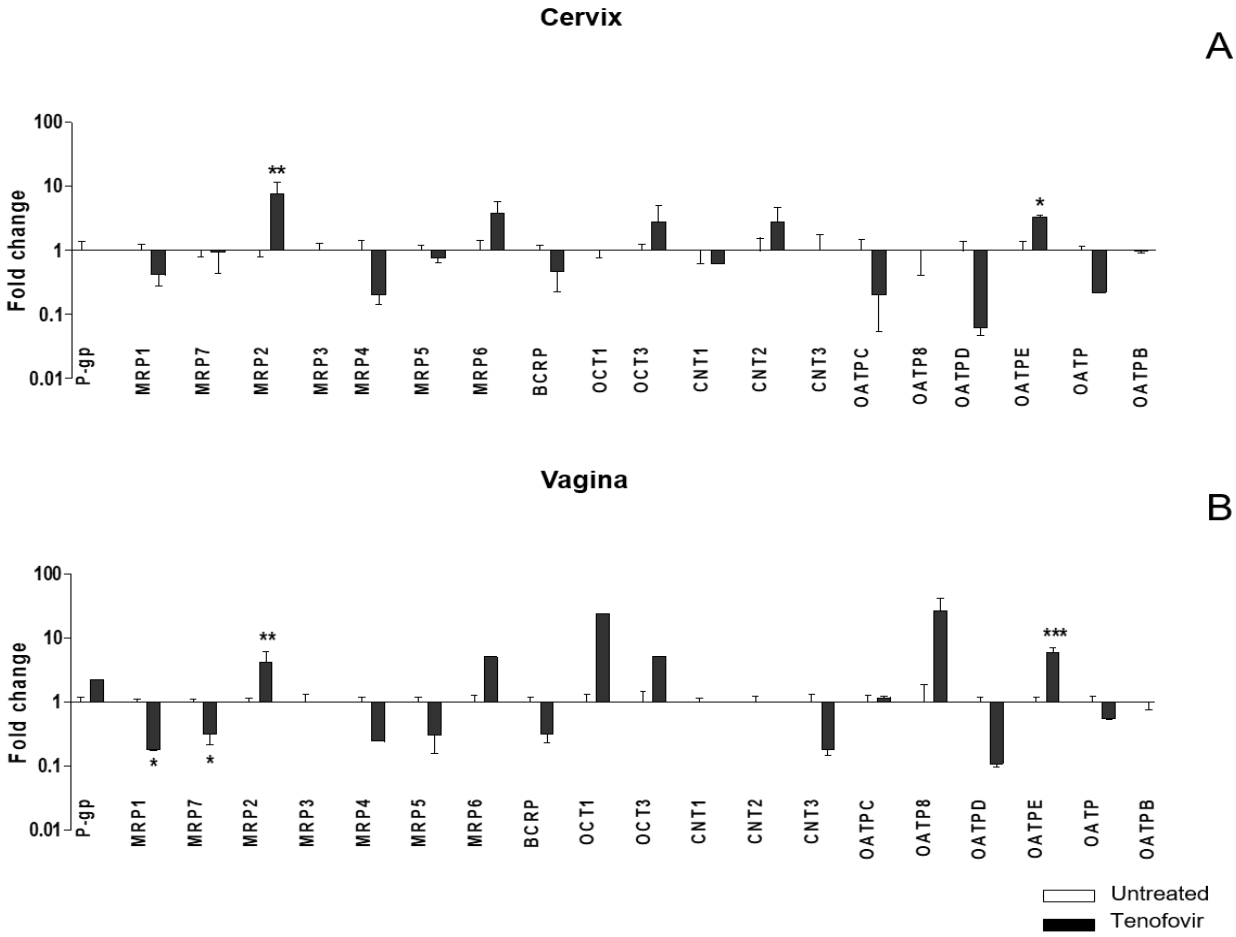
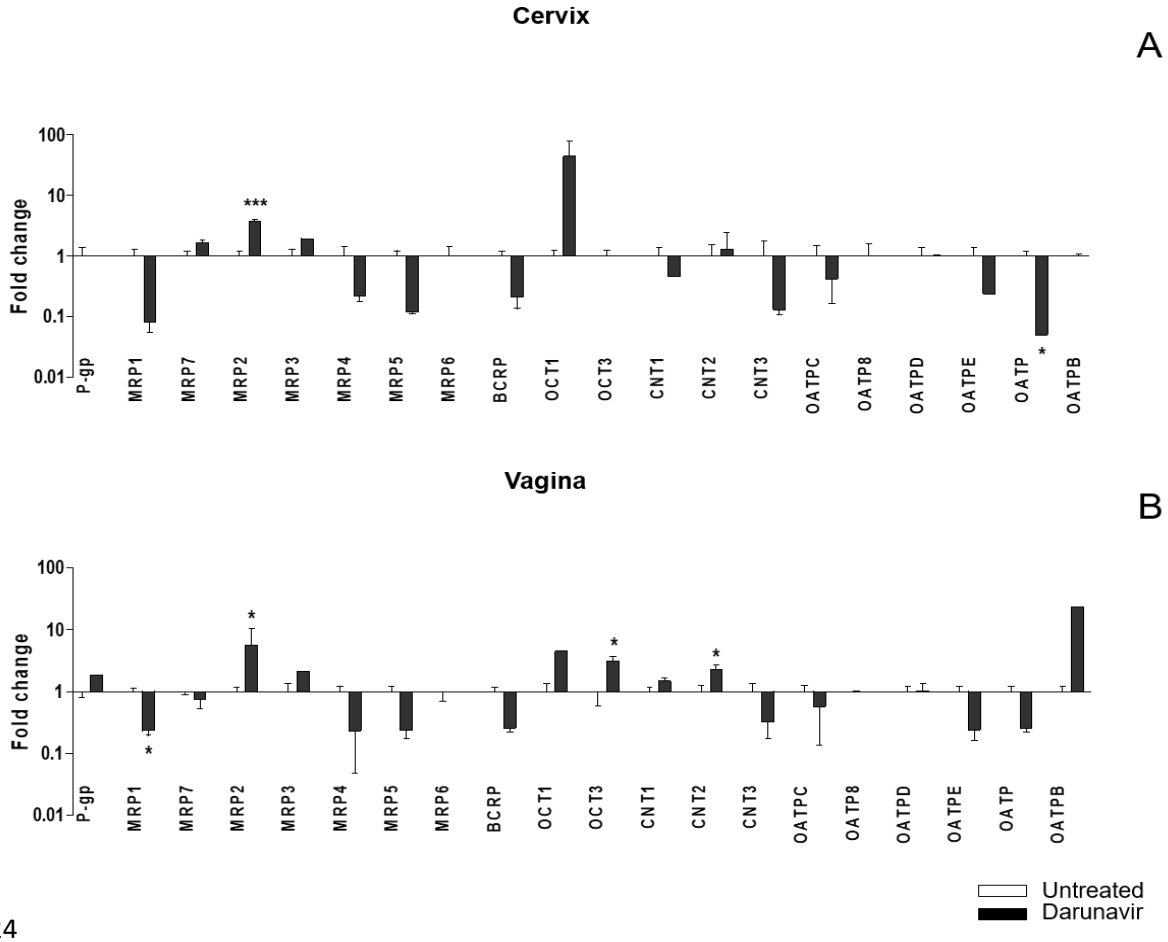


Figure 4 shows the effects of darunavir exposure on expression of drug transporters in the cervix and vagina. As observed in tenofovir-stimulated animals, the efflux transporter MRP2 was significantly upregulated by darunavir in both the cervix (3.8-fold, P=0.0004) and the vagina (5.7-fold, P=0.042). Darunavir also downregulated MRP1 (0.23-fold, P=0.021) but in vaginal tissue only. Amongst uptake transporters OATP was significantly downregulated by darunavir in the cervix (0.05-fold; P=0.035), while expression of OCT3 and CNT2 was significantly increased in the vagina (3.11 and 2.26- fold; P=0.041 and P=0.048 respectively) but not in the cervix.

As shown in Figures 3 and 4 expression of other key efflux transporters (MRP1, MRP4, MRP5, BCRP) was consistently downregulated by both drugs in cervical and vaginal tissue but changes in expression did not reach statistical significance.

No significant gene upregulation or downregulation was observed in animals exposed for 1 hour to either the tenofovir or darunavir dissolving films (data not shown).

Figure 4. Drug transporters gene expression in darunavir-treated macaque. Animals were vaginally treated with 1.7 mg darunavir dissolving film. Levels of total RNA extracted from vaginal and cervical biopsies at 24 hours were measured by quantitative PCR. Relative quantification was determined using the comparative Ct method with data normalized to three housekeeping genes (HPRT1, RPLP0, UBC) and calibrated to the average Ct of naïve animals (fold induction $2^{-\Delta\Delta Ct}$). Values of cervix **A**) and vagina **B**) are represented as mean ± SD of three samples carried out in triplicate on PCR runs. Statistical analysis was performed using *t*-Student's test (**P*≤0.05 and *** *P*≤0.001).

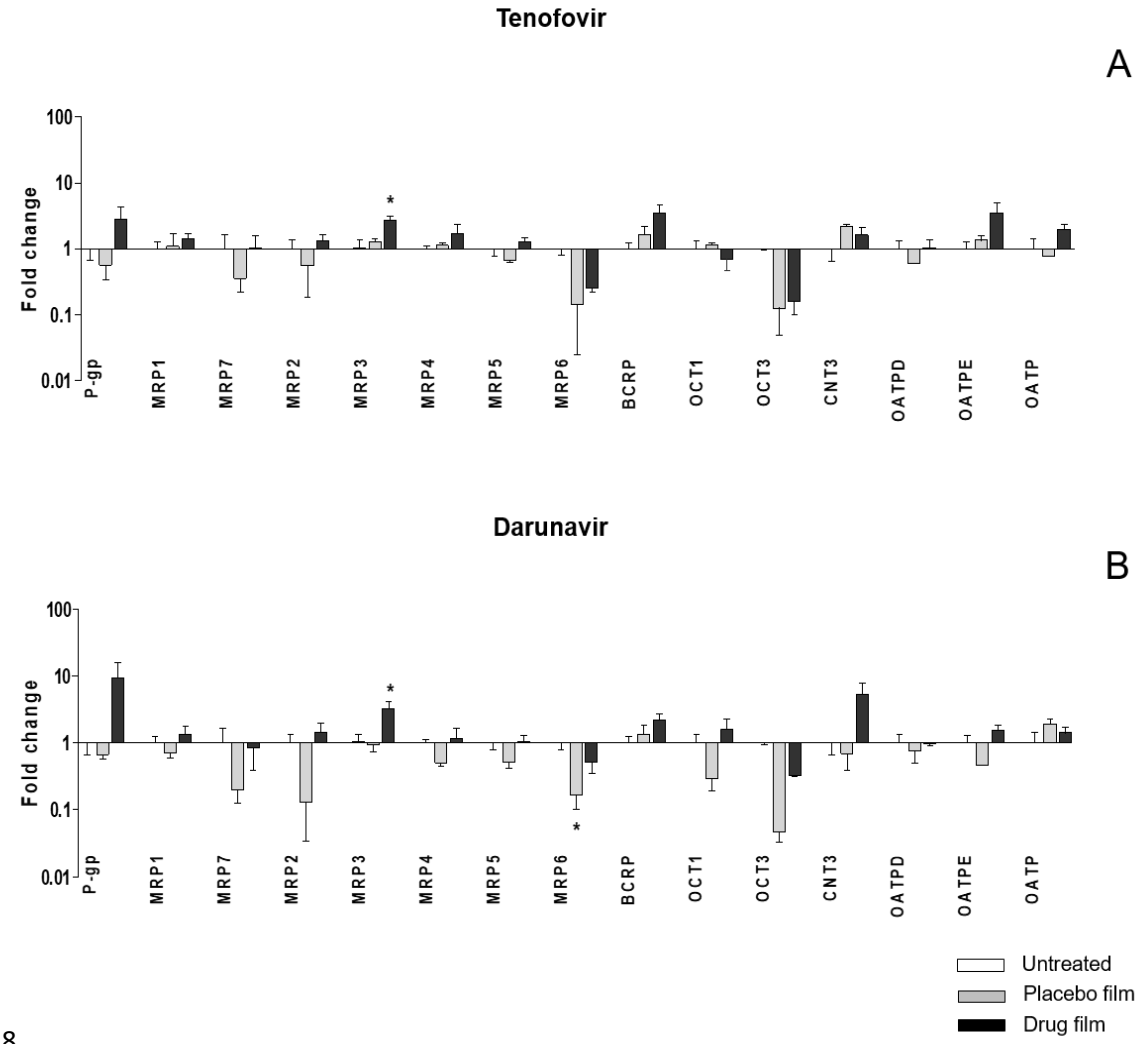


Effect of drug-free dissolving film vehicles on expression of drug transporters.

Potential effects of drug-free (placebo) dissolving films on drug transporter expression were not assessed in the macaque model due to ethical restrictions. To investigate whether placebo films may influence drug transporter expression, we used a previously described human cervico-vaginal cell line model (11). Drug transporter expression in VK2/E6E7 vaginal cell lines treated with film-released darunavir and tenovofir and respective placebo formulations

for 72 hours is shown in Figure 5. The effect of drug dissolving films versus untreated cells is shown in comparison with the effect of the respective placebo versus untreated cells. Exposure of cells to films loaded with darunavir or tenofovir resulted in significant upregulation of MRP3, as previously observed in VK2/E6E7 cells treated with free darunavir (11). The darunavir placebo had a downregulatory effect on all transporters with the exception of BCRP and OATP. However only downregulation of MRP6 reached statistical significance. None of the expression changes induced by the tenofovir placebo were statistically significant. No statistically significant changes in expression of drug transporters were observed in the uterine cell line HEC-1A, the endocervical cell line End1/E6E7 or the ectocervical Ect1/E6E7 treated with either the darunavir or tenofovir placebo (data not shown). There were no statistically significant differences between the effect of the tenofovir placebo and darunavir placebo on the expression of drug transporters in any of the cell lines (data not shown).

Figure 5. Drug transporters gene expression in tenofovir and darunavir dissolving film-treated VK2E6/E7 cell line. VK2E6/E7 cells were treated for 72 hours with tenofovir dissolving film (1 mM upon dissolution in culture medium) or respective placebo **A**) and darunavir dissolving film (250 μM upon dissolution in culture medium) or respective placebo **B**). The effect of drug dissolving films versus untreated cells is shown in comparison with the effect of the respective placebo versus untreated cells. Levels of total RNA were measured by quantitative PCR. Relative quantitation was determined using the comparative Ct method with data normalized to three housekeeping genes (HPRT1, RPLP0, UBC) and calibrated to the average of Ct of untreated control cells (fold induction $2^{-\Delta\Delta Ct}$). Values were represented as mean ± SD of five independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test (*P<0.05).



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461 **DISCUSSION**

462 Expression of drug transporters at the mucosal sites of HIV entry is likely to influence drug
463 retention and distribution of vaginally applied ARV-based microbicides (37). In this study we
464 showed that single vaginal administration of film formulations of tenofovir and darunavir
465 resulted in drug concentrations sustained over 24 hours in each macaque, that were well
466 above *in vitro* EC_{50} values, in vaginal fluid and tissues. They were also within the range of
467 concentrations that were previously shown to modulate drug transporter expression *in vitro*
468 (11). Large variations in the vaginal fluid drug concentrations between individual macaques
469 within each group (Figures 1A and 2A) were evident in this study. For tenofovir, at Tmax,
470 samples were less distributed (Cmax: 19.8$\pm$3.7mM) than at other time-points reflecting
471 significant variation in the rates at which drug concentrations decreased for individual

macaques. In contrast, for darunavir there was greater variation throughout the time course of the experiment. Within the group, Cmax for each macaque was reached at different time points and the rates at which drug concentrations decreased also varied. Darunavir with lower solubility in water (0.15 mg/ml) than tenofovir (1.8 mg/ml) (38) may diffuse less readily in vaginal fluid. Other studies involving alternative forms of drug administration for darunavir and other ARVs in macaque models have also shown large variations in drug levels in vaginal fluids (22, 39, 40). Although there were differences in the drug levels in other tissues between each pair of macaques at 1h and 24h post drug administration, these were of a much lower magnitude than those observed in vaginal fluid.

Previous *in vitro* studies, using a human colorectal cell line model indicate that uptake of darunavir and tenofovir is primarily by transcellular diffusion (41). Efflux transporters P-gp and MRP2 (to a lesser extent) reduced intracellular levels of darunavir but did not affect those of tenofovir. On the other hand, a permeability study in vaginal and T cell lines demonstrated energy-dependent intracellular accumulation of tenofovir, suggesting that the contribution of transcellular diffusion may be dependent of cell type (42). In this study, we show that P-gp is expressed in macaque vaginal and cervical tissue albeit at lower levels than in the corresponding human tissue. In contrast, MRP2 expression was confirmed in macaque vaginal and cervical tissues whereas in humans, expression was previously shown to be restricted to endocervical tissue [11]. At 1 hour post application of darunavir, drug concentrations in cervical and vaginal tissues were 3-4 logs higher than the EC_{50}. At 24 hours, there were further increases in darunavir concentration (3-4 fold) in tissues from near the middle and entrance of the vagina with little change elsewhere. Higher tissue concentrations of tenofovir at 1 hour may reflect the increased dose compared with that of darunavir. In addition, efflux of tenofovir may be reduced in comparison with darunavir since it is not a substrate for P-gp or MRP2 transporters (43). Intracellular conversion of tenofovir to tenofovir diphosphate, the active form of the drug, with median intracellular half-life of approximately 150 hours in human PBMCs (44) may further increase drug retention. Intracellular phosphorylation of tenofovir likely accounts for the apparent reduction in levels of tenofovir at 24 hours post application since, in this study, tenofovir diphosphate concentrations were not assessed.

Receptive anal intercourse among participants in clinical trials of vaginally applied microbicides has been proposed as a behaviour that may contribute to apparently reduced efficacy of the intervention (45). It is therefore of interest to assess the levels of ARV that

may accumulate in colorectal tissue following vaginal administration. In this study, the concentrations of darunavir in rectal tissues were more than 200-fold in excess of the EC_{50} and therefore may be sufficient to prevent against rectal challenge although there are no reports of testing darunavir for this purpose. Concentrations of tenofovir in rectal tissue were close to EC_{50} and may not be sufficient to prevent infection. A challenge study, in which pigtailed macaques were dosed vaginally with a gel formulation of tenofovir twice per week and were challenged rectally with SHIV also twice per week, showed delayed infection compared with the placebo group (4.5 fold reduced risk) but all animals were infected by the tenth challenge (46). Peak concentrations of tenofovir in rectal fluids reached approximately 63 μM and were 2 orders of magnitude lower than concentrations in vaginal fluid.

Drug transporter expression has been previously characterised in human cervicovaginal cell lines and tissue (9, 11, 47). In the present study we report expression of transporters in an established non-human primate model for pre-clinical development of vaginal microbicides (22). The qualitative expression profile of efflux and uptake drug transporter genes, associated with ARV transport, in macaque tissues was similar to that of human tissues although some MRP, CNT and SLCO transporters were expressed at low levels in macaque vagina but not in human tissue (11). Importantly, we confirmed resemblance of macaque cervicovaginal tissue to human tissue in that neither expresses OAT transporters, widely implicated in the uptake of tenofovir (9, 11). Some differences in transporter expression in macaque and human tissues were, however, also evident. As above, we observed significantly lower expression of P-gp in macaque compared to human cervicovaginal tissue that may result in decreased efflux of NRTIs (tenofovir disoproxil fumarate, abacavir) (48, 49) and PIs (darunavir, ritonavir, lopinavir, atazanavir, saquinavir, and indinavir) (50-52). Similarly, MRP5 (efflux of NRTIs (53)) and MRP1 (efflux transporter of PIs) were expressed at lower levels in macaques, In addition, OCT1-3 and CNT3 (implicated in uptake of NRTIs) as well as some SLCO uptake transporters (which influence uptake of PIs (8)), were expressed at lower levels in macaques. The different vaginal physiological pH in macaques (pH 7) and humans (pH 4.5) may contribute to the lower expression in simian tissue, although previous studies suggest increased functional activity of P-gp in acidic compared to neutral environments rather than pH-dependent differential expression (54, 55). The lower expression of certain efflux transporters in macaques may result in increased retention of NRTIs and PIs at the simian cervicovaginal mucosa compared to the human although this may be counteracted by the lower expression of uptake transporters. Thus cynomolgous

macaques represent a similar but not identical model to humans for investigation of pharmacokinetics of vaginal dosing.

Our findings that P-gp, BCRP and MRP4 are expressed in tissues of cynomolgous macaques are in keeping with a previous report demonstrating expression of these key efflux transporters in the lower genital tract of female pigtailed macaques (47).

Protein expression determination would be desirable to support our findings from quantitative mRNA analyses. Several studies demonstrate close agreement between gene expression and proteomics measurements of the drug transporters investigated in this study (18). For example, studies of protein and mRNA expression showed significant correlations between protein and mRNA levels for BCRP and OCT3 in the human small intestine (16) and P-gp, MRP4 and BCRP in both macaque and human cervicovaginal tissue (47). The same positive correlation was identified in a study of the expression of a range of efflux and uptake transporters in the human cervicovaginal tract investigated in this study (9). Characterisation of drug transporter distribution at the plasma membrane and within the stratified epithelium as well as studies of drug transporter activity will be useful to predict their role in overall drug transport across the epithelial barrier. In this respect, Zhou *et al* observe a preferential cytoplasmic distribution of P-gp and MRP4 in epithelial cells of macaque ectocervix and vagina but no clear difference in signal intensity between different layers of the epithelium (47). Assays based on selective inhibition of drug transporters could provide useful data on the net effect of drug transporter activity versus passive transcellular or paracellular transport on mucosal disposition of vaginally-delivered combinations of NRTIs and PIs.

We previously measured the modulatory effect of liquid forms of tenofovir, darunavir and dapivirine on expression of drug transporters in a panel of cervicovaginal cell lines. In those studies stimulation of cell lines with darunavir resulted in significant upregulation of various MRP transporters but not MRP2, P-gp and BCRP (11). Amongst uptake transporters CNT3, OCT3 and SLCO transporters were upregulated by darunavir but not consistently amongst vaginal, ectocervical and endocervical cell lines (11). Stimulation with tenofovir resulted only in downregulation of MRP5 in vaginal but not cervical cell lines (11). In the present study we have shown that the modulatory effect of film-released darunavir and tenofovir *in vivo* was overall very different compared to the effect of soluble drugs in cell lines. In particular, we observed that MRP2 (efflux transporter for which both NRTIs and PIs may be substrates) was the only MRP transporter significantly upregulated by both film-released

darunavir and tenofovir in macaques. In addition, we observed significant downregulation of the SLCO transporter OATP and upregulation of CNT2 by darunavir which was not observed in cell lines stimulated with soluble drug. Likewise, the significant upregulation of OATPE induced by film-released tenofovir was not observed *in vitro* (11). Upregulation of OCT3 by film-released darunavir in macaque vaginal tissue was the only change seen in VK2/E6E7 cells stimulated with soluble darunavir (11). Expression changes of other uptake transporters that were up- or down-regulated in our previous *in vitro* study did not reach statistical significance *in vivo*.

Factors relating to both the drug formulation and the experimental model may explain differences in stimulatory/inhibitory activity of drug transporter expression by darunavir and tenofovir observed in the two studies. To determine the role of the drug-free vehicle on drug transporter expression we tested the effect of the placebo films on the VK2/E6E7 cell line. The darunavir placebo formulation downregulated MRP2 and upregulated OATP but not at statistical significance. This finding demonstrates that the effect of the drug vehicle is unlikely to account for the significant increase in MRP2 and decrease of OATP observed in animals treated with film-released darunavir. Similarly, the effect of the tenofovir placebo on drug transporter expression did not explain the expression changes induced by film-released tenofovir administered to macaques. Nonetheless, the mostly inhibitory effect on expression by the placebo preparations may partly explain the overall lower stimulatory activity on drug transporter expression by the drug-dissolving films *in vivo* when compared to the effect of drugs in the liquid form *in vitro*, particularly in the case of darunavir (11). Excipients such as polyethyleneglycol and Peocol, both included in the films used in this study, have previously been shown to downregulate P-gp expression (56, 57). Our findings indicate that some degree of downregulation may extend to other transporters pointing to the potential beneficial effect on tissue penetration of the microbicide vehicles tested here. Further, differences in expression of drug transporters observed *in vivo* and *in vitro* are likely due to the effect of physiological environmental factors such as pH, mucous, microbiota and the oestrous cycle. Indeed, a murine study demonstrated that expression of efflux transporters in the cervix and vagina can be modulated by the oestrous cycle (17). While the direct role of vaginal microbiota on modulation of drug transporters is yet to be demonstrated, imbalances of the vaginal microbiome have been associated with decreased concentrations of ARV drugs retrieved in cervicovaginal lavage (58). This evidence suggests that, where possible,

physiological environmental factors must be taken into account in cell line-based kinetic models of drug transport.

CONCLUSIONS

The comparative analyses of drug transporter expression in human and macaque tissue provide mechanistic information that will aid interpretation of PK/PD data in the context of pre-clinical studies and clinical studies. The finding that concentrations of tenofovir and darunavir released from dissolving films were well above the EC_{50} values both in vaginal fluid and vaginal tissues over 24 hours is important in the context of development of applicator-free and leakage-free vaginal formulations. Data on the modulatory effect of film-released tenofovir and darunavir on drug transporter expression will inform strategies to facilitate drug penetration across the epithelial barrier and increase distribution at target cells. This could be achieved by selective inhibition of efflux transporter MRP2 to increase absorption of mucosally-delivered NRTIs and PIs. ARV drugs may be also strategically combined to achieve the desired effect on drug transporters. For example, the inhibitory effect of tenofovir on OATP may aid absorption of PIs by diminishing potential intracellular accumulation of these hydrophobic drugs in the epithelium.

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SUPPORTING INFORMATION

Supplemental figure 1. Darunavir release in lymph nodes

Supplemental figure 2. Tenofovir release in lymph nodes

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