



Drug transporter gene expression in human colorectal tissue and cell lines: modulation with antiretrovirals for microbicide optimization

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1	Drug Transporter Gene Expression in Human Colorectal Tissue and Cell Lines:								
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Running Title: Drug transporters for antiretroviral drugs in human colorectal tissue.

22 Key Words: Drug transport, gene expression, colon, Caco-2 cells, antiretroviral

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28 SYNOPSIS

Objectives To comprehensively assess mRNA expression of 84 drug transporters in human colorectal biopsies and 6 representative cell lines. To investigate the alteration of drug transporter gene expression after exposure to three candidate microbicidal ARV drugs, (tenofovir, darunavir and dapivirine) in Caco-2 cells. The outcome of the objectives informs development of optimal antiretroviral (ARV)-based microbicidal formulations for prevention of HIV-1 infection.

Methods Drug transporter mRNA expression was quantified from colorectal biopsies and cell lines by quantitative real-time PCR. Relative mRNA expression was quantified in Caco-2 cells
 after induction with ARVs. Data was analysed using Pearson's correlation coefficients (r),
 hierarchical clustering and principal component analysis (PCA).

39 Results Expression of 58 of the 84 transporters was documented in colorectal biopsies, with CNT2, P-gp and MRP3 being the highest expressed. No difference was noted between 40 41 individual subjects, when analysed by age, gender or biopsy site (rectum or recto-sigmoid) (r=0.95-0.99). High expression of P-gp and CNT2 proteins was confirmed by 42 immunohistochemical staining. Similarity between colorectal tissue and cell-line drug 43 44 transporter gene expression was variable (r = 0.64-0.84). PCA showed distinct clustering of 45 human colorectal biopsy samples with the Caco-2 cells defined as the best surrogate system. Induction of Caco-2 cell-lines with ARV drugs suggests that darunavir-based microbicides 46 incorporating tenofovir may result in drug-drug interactions likely to affect distribution of 47 individual drugs to sub-epithelial target cells. 48

49 **Conclusions** These findings will help optimise complex formulations of rectal microbicides to
50 realise their full potential as an effective approach for pre-exposure prophylaxis against HIV51 1 infection.

52

54 **INTRODUCTION**

The Human immunodeficiency virus-1 (HIV-1) pandemic is in its third decade and current 55 strategies to prevent acquisition and dissemination of this infection are focused on oral 56 antiretroviral (ARV) drugs as pre-exposure prophylaxis.¹ ARV based vaginal microbicides on 57 the other hand have shown variable efficacy levels in randomized controlled trials, with 58 adherence being the critical factor for determining their efficacy.²⁻⁵ There is also a definite 59 clinical need in modulating microbicides to prevent HIV-1 infection via the anorectal route, 60 which has around 20 times higher transmission risk per exposure compared to the vaginal 61 62 route.^{6,7} In addition to exploiting breaches within the epithelial barrier and potential interaction with intraepithelial migrating cells - the main mechanisms implicated in HIV-1 transmission 63 across the vaginal mucosal barrier – HIV-1 can cross the rectal epithelium by transcytosis 64 through epithelial cells⁸ and M cells⁹, and opening of tight junctions.¹⁰⁻¹² The efficacy of rectal 65 66 microbicides has been assessed in non-human primate models and in ex-vivo colonic explant culture systems.^{8, 9, 13, 14} This preclinical evaluation of potential microbicides in these models 67 is essential as the pharmacokinetics of ARV drugs, more specifically Dapivirine, has been 68 shown to be dissimilar in colonic and vaginal tissues of human volunteers, which was 69 70 postulated to be due to differences in the local expression of the cytochrome P450 enzymes.¹⁵

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The pharmacokinetics of topically administered ARV drugs in the colon is likely dependent not 72 only on their metabolism by the cytochrome P450 enzyme system but by the expression of 73 drug transporters in the colorectal epithelium.^{16, 17} The expression of drug transporters may 74 75 play a central role in not only determining the mucosal concentration of ARV microbicidal drugs being used as a combination but also contributing to the complex drug-drug interactions 76 between the constitutive drugs.¹⁸ The major efflux transporters are the ATP Binding Cassette 77 (ABC) transporters whilst the Solute Carrier (SLC) and Solute Carrier Anion (SLCO) 78 transporters are largely responsible for uptake of drugs. Understanding of the interplay 79 between different ARV drugs and drug transporters expressed in the colorectal epithelium will 80

81 help design effective microbicide combinations. This in turn will finally determine the extent of mucosal disposition and active concentration of the ARV drugs in the CD4+ T cells which are 82 the primary targets and reservoirs of HIV-1 infection.¹⁹ The analyses of selective subsets of 83 drug transporters in the colon have documented high expression of MRP3, P-glycoprotein (P-84 gp), MCT1 and BCRP.²⁰⁻²² The interaction of various classes of ARV drugs with these and 85 other important drug transporters have been well documented in the small intestine, liver and 86 kidney but not in the colorectal epithelium.¹⁸ Three ARV drugs which are currently being tested 87 as potential microbicides include tenofovir, darunavir, and dapivirine.²³ However, most of the 88 89 studies utilise these drugs in formulations for vaginal application and it is critical that their safety and efficacy as rectal microbicides is assessed separately. 90

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92 The physiological expression of colorectal drug transporters has been largely interpreted from surgical resection specimens as well as diagnostic endoscopic biopsies.^{20, 24} Understanding 93 of the xenobiotic pathways undertaken by ARV drugs and their interaction with drug 94 95 transporters requires a robust in vitro experimental model. Colorectal primary cell cultures 96 have been used for this purpose but are affected by rapid loss of differentiation of the cells which does not permit longer induction studies with ARV drugs.^{25, 26} As a result, most of our 97 knowledge is based on information from colorectal cell lines.^{27, 28} However, mRNA expression 98 99 of drug transporters in these organotypic cell lines does not correlate with that observed in human colonic tissue.^{20, 22} Human colorectal explant-based models currently used to pre-100 clinically evaluate the antiviral activity of candidate microbicides may be a suitable model for 101 evaluating ARV interactions with drug transporters, although this may be limited by short-term 102 viability.9, 13, 14 103

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The pharmacokinetics of ARVs as topical microbicidal agents in the colorectal epithelium has not been fully elucidated. There have been several studies which have looked at a subset of both ABC and SLC transporters in the colon compared with other organs and organotypic cell lines, but the changes of gene expression on exposure to ARV's have not been documented.²⁰⁻²² The aim of the current study was to provide a comprehensive
characterisation of the physiological expression of 84 drug transporter genes in the colorectal
epithelium. This was coupled with induction studies of a surrogate colorectal cell line to outline
the functional consequences of ARV exposure on drug transporter gene expression.

113

114 MATERIALS AND METHODS

115 **Tissue samples**

Human colorectal biopsies were obtained from healthy subjects undergoing colonoscopy at 116 Aberdeen Royal Infirmary (UK) as part of the Scottish Colorectal Cancer Screening Program. 117 Ethical approval for this study was obtained by the North of Scotland Research Ethics Service, 118 UK (reference numbers 09/SO802/106 and 12/NS/0061). Subjects were recruited to the study 119 after obtaining written informed consent according to the World Health Organisation guidelines 120 for good clinical practice (GCP) and the local Research Ethics Committee policies. Biopsy 121 122 samples were collected from subjects whose colonoscopy was found to be normal. The drug list of the participants was scrutinised prior to their inclusion into the study. Subjects were 123 excluded if they had received systemic antibiotics or steroids in the three months prior to their 124 125 colonoscopy or had been on any immunosuppressive drugs at any time. Subjects were also 126 excluded if they had any major co-morbidity. The subjects recruited were all healthy and the majority (62%) were on no concomitant medications for the last three months. The rest 38% 127 were on routine anti-hypertensives, statins, paracetamol or aspirin, which is expected in the 128 129 study cohort chosen. Paired samples from two standard anatomical sites, rectum and rectosigmoid, were collected and four mucosal punch biopsies were obtained from each site. 130 131 Biopsies were removed from forceps and placed in sterile 1.5mL tubes, immediately snapfrozen in liquid nitrogen and were subsequently stored at -80°C until RNA extraction. 132 Colorectal biopsies for drug stimulation studies were collected from healthy subjects and 133 specimens were immediately placed in DMEM (Sigma, Dorset, UK) containing 10% heat-134 inactivated FCS (HyClone Laboratories, Utah, USA), 2% L-glutamine (Sigma) and antibiotics 135

(100U penicillin, 100 µg of streptomycin, and 50 µg of gentamicin/mL, Sigma). For
immunohistochemical studies normal colorectal biopsies were obtained from the NHS
Grampian Biorepository (ethics reference number 11/NS/0015; tissue bank request number
TR000005).

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141 Cell lines and culture methods

Human colorectal (Caco-2 and HT-29) and rectal (SW1463) adenocarcinoma cell lines were 142 purchased from Health Protection Agency culture collections (HPA, Salisbury, UK). Caco-2 143 144 cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Sigma) containing 10% FCS, 1% nonessential amino acids (Sigma), and 2% L-glutamine. Cells were seeded at a 145 density of 1 × 10⁶ cells/ well on 12 mm Transwell® Permeable Supports with 0.4µm pore 146 polycarbonate membrane insert and 1.12 cm² growth areas (Corning Costar, Cambridge, MA, 147 148 USA). Membrane filters were placed in a 12 mm well with 1.5 and 0.5 mL of culture medium in the baso-lateral and apical compartments, respectively. The medium for the Transwell® 149 plates was supplemented with 0.1% gentamicin. Cells were cultivated over 21 days at 37°C 150 in an atmosphere of 5% CO₂. Medium was changed from both the compartments every second 151 152 day. Transepithelial Electric Resistance (TEER) was measured using EVOM² epithelial voltohmmeter and STX2 electrodes (World Precision Instruments, Hertfordshire, UK) and 153 TEER values of approximately 2000 ohms/cm² after 21 days were accepted as an indication 154 of an intact monolayer. HT-29 cells were cultured in McCoys 5A modified medium (Sigma), 155 supplemented with 10% FCS and 2% L-glutamine and incubated at 37°C with 5% CO₂. HT-156 29 cells were routinely sub-cultured by trypsinization using trypsin (0.05%)-EDTA (0.02%) 157 solution (Sigma), and were seeded at a density of 2×10^6 cells per 75-cm² flask. SW1463 cells 158 159 were grown in L15 medium (Sigma) with 10% FCS and 2% L-glutamine and incubated at 37°C 160 atmospheric air with no CO₂.

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162 Anti-retroviral compounds

Tenofovir was provided by Gilead Science (Foster City, CA, USA). Darunavir was provided by
Janssen R&D Ireland (Cork, Ireland). Dapivirine was purchased from Selleckchem (Suffolk,
UK). Stock solutions of dapivirine and darunavir were dissolved in DMSO (Sigma) and
tenofovir was dissolved in sterile PBS. The maximum DMSO concentration in the induction
assays never exceeded 1% (v/v).

168

For preparation of darunavir-based dissolving films micronized darunavir (0.57 %) was added 169 to a mixture of tween 80 (0.55%, Spectrum Chemical) and water (91.499 %, Ricca) and 170 homogenized for 10 minutes using a probe homogenizer. PEO-10 (1.10 %, Dow) and PEO 171 WSR301 (0.055 %, Dow) were added and mixed for 30 minutes. PEG 1000 (0.916 %, EMS) 172 was then added and mixed for another 30 minutes using a mechanical stirrer. Once 173 174 homogeneous, HPMC E50 (5.310 %, Ashland Chemical) was added to the above dispersion 175 and stirred by mechanical mixer for an additional 120 minutes, followed by bath sonication of the dispersion for 30 minutes to remove any entrained air. The dispersion was coated onto 176 release paper at slow speed using a Coatema Easycoater, set for 1400 µm wet thickness, and 177 then dried at 37°C. For preparation of tenofovir-based dissolving films, tenofovir (1.89 %) was 178 179 added to a mixture of glycerin (3.33 %, Fisher) and water (53.37%, Ricca) and homogenized for 2 minutes using a probe homogenizer. NaOH at 1 M (6.50 %, Fisher) was added until 180 tenofovir was completely dissolved. A premixed solution of propyl paraben (0.01 %, 181 Spectrum)/PECOL (0.83 %, Gattefosse) and alcohol (28.07 %, Spectrum) was added to the 182 tenofovir solution; this solution was covered to minimize evaporation. PEO 205 (1.00 %, Dow) 183 was added to the above final solution and mixed using a mechanical stirrer for 10 minutes. 184 Once dissolved, Benecel E50 (5.00 %, Ashland) was added and mixed for another 60 minutes, 185 followed by bath sonication of the dispersion for 30 minutes to remove any entrained air. The 186 dispersion was coated onto release paper at slow speed using a Coatema Easycoater set for 187 1400 µm wet thickness, and then dried at 37°C. 188

189

190 Cytotoxicity assays

Alamar Blue® cell viability assay (Life Technologies, Paisley, UK) was used to determine the 191 tolerability of Caco-2 cells to the three drugs and DMSO vehicle. Caco-2 cells were seeded in 192 96-well plates and allowed to adhere for 48 hours. Medium was replaced with serial dilutions 193 194 of fresh medium containing dapivirine (0-100µM), darunavir (0-750µM),tenofovir (0-5mM) or DMSO (0-1%). After 24, 72 and 168 hours at 37°C, 10% alamar Blue® was added to each 195 well. Following 4 hour incubation, fluorescence was monitored at 530 nm excitation wave 196 lengths and 590 nm emission wave lengths on a Synergy™ HT Multi-Detection Microplate 197 198 Reader (BioTek).

199

200 Stimulation of Caco-2 cells with ARV drugs

201 The uppermost tolerable concentrations of the three drugs which could be solubilised in an appropriate vehicle (10µM dapivirine, 250µM darunavir and 5mM tenofovir) were added to the 202 apical surface of the Caco-2 cell layers separately in different wells. For each experiment an 203 appropriate control well was included (cells with 1% DMSO as control for darunavir and 204 dapivirine stimulation and cells with media alone for tenofovir stimulation). The cells induced 205 206 with drugs were then incubated in a 37°C incubator with 5% CO₂ for 24-168 hours. Additionally, darunavir, tenofovir and respective placebo dissolving films (1.5 cm²) were 207 subdivided into 6 equal sections using a ruler for accurate measurement and were cut using 208 a sterile scalpel precisely. Individual film sections were then added to the apical surface of the 209 confluent 21 day Caco-2 cell layers in Transwells and incubated at 37°C with 5% CO₂ for 72 210 hours. After the incubation period cells were harvested from the Transwell® inserts for RNA 211 extraction. All stimulation experiments were done as three biological repeats. 212

213

214 **Explant culture**

After collection, biopsies were maintained at 4^oC for transportation to the laboratory and were processed within 1 hour of collection in complete media as described earlier. In the laboratory, biopsies were washed and explant culture was done following methods published previously.¹³

218 Biopsies were cultured and supported on pre-soaked gel foam rafts (1 cm², 7mm thick; Wellbeck Pharmaceuticals, USA) at the air-media interface in 24-well plates containing 300 219 uL of media. They were incubated in blank media for 24 hours in a humidified atmosphere 220 221 with 5% CO₂ and then either snap-frozen in liquid nitrogen for RNA extraction or formalin-fixed 222 for histological examination. For the latter, eight biopsies collected from a single subject were 223 incubated at four different experimental conditions in pairs. One pair of biopsies were 224 immediately formalin fixed on arrival in the lab to act as controls, two biopsy samples were 225 incubated in media alone, two with media containing 1% DMSO vehicle and two pieces with 226 one of the ARVs (10µM DPV) for 24 hr on gel foam rafts. Formalin-fixed tissues were 227 embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E).

228

229 Stimulation of colorectal explants with ARV drugs

230 From each individual subjects, 2 biopsies were stimulated with 10uM Dapivirine, 2 with 250uM Darunavir, 2 with 5mM Tenofovir and 2 with appropriate controls (media or 1% DMSO).

231

Stimulated explants were cultured in pairs on pre-soaked gel foam rafts and incubated for 24 232

- hr in a humified atmosphere with 5% CO2 and then snap frozen in liquid nitrogen for RNA 233
- 234 extraction.
- 235

Isolation of RNA and cDNA synthesis 236

Total RNA was extracted from colorectal biopsies using a combination of the TRIzol reagent 237 (Invitrogen, UK) and the Qiagen Rneasy Micro kit (Qiagen, Manchester, UK) following the 238 manufacturer's recommendations. RNA was extracted from HT-29 and Caco-2 cell cultures 239 using the Qiagen Rneasy Mini kit (Qiagen) following the manufacturer's recommendations. 240 241 DNase treatment was performed on all RNA samples to remove genomic DNA contamination. RNA preparation was quantified using a Nanodrop ND-1000 UV spectrophotometer (Thermo 242 Fisher Scientific, MA, USA). The integrity of the RNA was confirmed for all samples using 243 Agilent 2200 TapeStation system. Complementary DNA (cDNA) was prepared from total RNA 244 by using SuperscriptTM First-Strand Synthesis System for real-time PCR (Life Technologies, 245

- Paisley, UK). For rectal adenocarcinoma cell lines SW837, HRA-16 and HRT-18, total RNA
 was directly purchased from HPA.
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249 **Relative mRNA expression analysis**

250 Real Time quantitative PCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA) for the analysis of 84 drug transporter genes 251 and 12 housekeeper genes using TaqMan® Array 96-well Fast plates for Human Drug 252 253 Transporters (Life Technologies). Quantitative PCR was performed in a final reaction volume of 10uL per gene containing 20ng of RNA converted to cDNA and TaqMan® Gene Expression 254 255 Master Mix (Life technologies) according to the manufacturer's instructions. The cycling conditions were: stage 1 (50°C, 2 min); stage 2 (95°C, 10 min); stage 3, 40 times (95°C, 15 s, 256 followed by 60°C, 1 min). For quantifying the effect of formulated ARV films on drug transporter 257 258 gene expression in Caco-2 cells, Single tube Taqman® gene expression assays were used for a selected subset of drug transporter genes known to be relevant for ARV transport (Pg-p, 259 BCRP, MRP1-5, MRP10, CNT2, ENT2, LAT2, OATPE and OATPD)¹⁸ using the same cDNA 260 concentration and PCR cycling conditions as the arrays. The relative gene expression levels 261 were determined using the comparative CT method ($\Delta\Delta$ CT method). ²⁹ The cut-off CT was 262 set at 35 cycles for all analysis. From the initial panel of twelve housekeeper genes, HPRT1, 263 PGK1 and PPIA were selected as endogenous controls, as determined by SD scores which 264 is an indicator of consistent expression across all samples and is calculated using geometric 265 averaging.³⁰ Human universal reference total RNA (Clontech Laboratories, CA, USA), 266 comprising of a standardized mixture of total RNA from a collection of adult human tissues, 267 was used as calibrator as previously described.²⁷ The mRNA levels of each transporter in this 268 RNA reference was arbitrarily set at the value of 1 unit and data for colorectal tissue and cell 269 lines were expressed in arbitrary units comparatively to the standardised RNA reference. 270 271 DataAssist® software (Applied Biosystem) was used for analysis of the quantitative PCR data. The relative quantification (RQ) values were expressed as mean and standard deviation. An 272 273 arbitrary classification system was assigned to the data, designating relative expression levels

>2 as high mRNA expression, levels between 2 and 1 as moderate mRNA expression, levels
between 1 and 0.1 as low mRNA expression and levels below 0.1 as unexpressed. The
variable expression group included genes which were expressed in a proportion of subjects
(33% -92% subjects) but not expressed in others with the mean RQ in the group ranging from
0.15 to 1.96. Ranking of the genes were then done according to their mean expression and
fold variation.

280

281 Immunohistochemistry

282 Colorectal biopsies taken from the initial cohort of subjects were utilized for gene expression analysis and showed no inter-subject differences. To confirm localization of the predominant 283 284 drug transporters, biopsies for immunohistochemistry were obtained from new recruits from the same screening list. Formalin-fixed and paraffin-embedded biopsy samples were cut into 285 286 4 µm sections and mounted on glass slides. The slides were deparaffinised in xylene and hydrated in graded ethanol series. Heat induced antigen retrieval of epitopes was done by 287 288 microwaving the slides for 20 minutes fully immersed in citrate buffer (pH 6.0). Slides were then stained with primary antibodies for ABCB1/P-gp, clone JSB-1 (Abcam, Cambridge, UK; 289 290 1:40 dilution), SLC29A2/ENT2, clone EPR11674 (Abcam,; 1:200 dilution), SLC289A2/CNT2 (Abcam; 1:40 dilution) and chromogranin A, clone DAK-A3 (Dako; 1:200 dilution) followed by 291 peroxidase conjugated goat anti-rabbit/mouse secondary antibody (Dako EnVision™ FLEX 292 Detection system, Dako, Ely, UK). DAB (3, 3'-diaminobenzidine) was used as the substrate 293 chromogen. Staining was performed using the DAKO AutoStainer (Dako) as previously 294 published.³¹ Slides were washed in water and counter stained with haematoxylin. Normal 295 human liver sample was used as a positive control for P-gp, ENT2 and CNT2 whereas normal 296 human pancreas sample was used as positive control for chromogranin A. For negative 297 control, the primary monoclonal antibody was omitted from the immunohistochemical 298 299 procedure and replaced with antibody diluent.

300

301 Statistical analysis

302 Pearson's product moment correlation (r) was used to look for differences of gene expression using ΔCT values amongst groups and correlation was observed at the significance level 303 p = 0.05. Hierarchical clustering was done for all genes using Pearson's correlation. Principal 304 component analysis (PCA) was performed to elucidate differences between colorectal biopsy 305 306 samples and various cell lines using using normalized expression levels (Δ CT).²¹ Scatter plot was generated using principal component 1 and 2. All mRNA expression data are presented 307 as means ± standard deviation and Independent (unpaired) samples t test was used to 308 demonstrate differences of mRNA expression. The unpaired samples t-test was used as the 309 310 mRNA expression data were collected from two independent and identically distributed sets of samples, one from each of the two populations being compared. Statistical analysis was 311 312 performed using SPSS 21 (IBM Corp; Armonk, NY) software.

313

314 **RESULTS**

315 Demographic characteristics of subjects

316 A total of twenty-six healthy subjects were recruited for this study. Biopsy samples from twelve healthy subjects (EU3 to EU10, EU12, EU13, EU14 and EU16), of whom six were male and 317 six female, were used to study the drug transport gene mRNA expression in normal colorectal 318 319 tissue. The mean age was 58.6 ± 7.4 years (range 50-70 years). Biopsies from seven more subjects were obtained from the NHS Grampian biorepository to study protein expression of 320 highly expressed colorectal drug transporters (EU42 to EU47 and EU49, mean age 57.9 ± 8.4 321 years, six male and one female). Seven further subjects (EU24, EU25, EU48, EU54, EU55, 322 EU56 and EU60, mean age 64.6± 3.5 years, 3 male and 4 female) were recruited for tissue 323 explant culture. One pair of biopsies from EU24, EU25, EU48 and EU54 were used for 324 baseline drug transporter gene expression assessment after 24 hours explant culture in 325 medium alone, biopsies from EU54, EU55 and EU60 were used for ARV stimulation studies 326 327 of explants and EU56 was used for H&E staining.

329 Drug transporter gene expression in colorectal biopsies

Out of the 84 drug transporter genes analysed 52 were expressed, 26 were not expressed 330 and 6 had variable mRNA expression in colorectal tissue. SLC28A2/CNT2 was the most 331 expressed uptake transporter in this study (RQ 25.8 \pm 21.6) followed by efflux transporters 332 333 ABCB1/P-gp (RQ 4.8± 2.7) and ABCC3/MRP3 (RQ 3.9 ± 1.6). The rank order of the highest expressed efflux and uptake drug transporters in colorectal tissue were ABCB1/P-gp > 334 ABCC3/MRP3 > ABCG2/BCRP > ABCD3 (RQ 4.8± 2.7, 3.9±1.6, 2.5± 1.3 and 2.3 ± 0.6 335 respectively) and SLC28A2/CNT2 > SLC16A1/MCT1 > SLC29A2/ENT2 > SLC28A3/CNT3 336 (RQ 25.8 ± 21.6, 2.8 ± 2.5, 2.4 ± 0.8 and 2.3 ± 1.5 respectively) (Tables 1 and 2, Supplementary 337 Table 1). There was no mRNA expression documented for the important efflux transporter 338 339 ABCC2/MRP2, uptake transporters SLC22A1-3 (OCT1-3) and kidney specific SLC22A6-8 (OAT1-3) drug transporters. The mean and standard deviation of RQ values of all the 84 drug 340 341 transporters are enumerated in Supplementary Table 1. The differences between gene expressions of these 84 genes were compared between the individual subjects and assessed 342 with respect to gender and site of biopsy. No significant difference was noted in mRNA 343 expression of the drug transporter genes between individual subjects (r = 0.98-0.83, 344 345 p<0.0001), when stratified according to sex (r = 0.98-0.95, p<0.0001) or by colorectal biopsy sites (rectum and recto-sigmoid; r = 0.99-0.96, p<0.0001). 346

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348 Drug transporter protein expression in colorectal biopsies

Immunohistochemical staining was performed on colorectal biopsy samples from seven 349 subjects for the ABCB1/P-gp, SLC28A2/CNT2 and SLC29A2/ENT2 proteins as they were 350 found to be highly expressed on the mRNA analysis. Immunohistochemistry analysis for other 351 highly expressed drug transporters was limited by the unavailability of specific and well 352 characterised antibodies. Immunohistochemistry of colorectal biopsies for the ABCB1/P-gp 353 protein showed uniform, strong staining of the surface microvillus border (Figure 1a) 354 355 correlating with mRNA expression of P-gp in the colorectal tissues (Table 1). The staining 356 pattern suggests that ABCB1/P-gp protein expression is predominantly in the luminal plasma

membrane of epithelial cells as previously documented ³². SLC29A2/ENT2 staining was 357 prominent in the epithelium and sub-epithelial cells (Figure 1b). The localization of this protein 358 appeared to be primarily in the nucleus which is consistent with this transporter being involved 359 in transporting purine and pyrimidine analogues.³³ Staining for SLC28A2/CNT2 showed 360 361 sparse staining of cells in the epithelium, which appeared separate and distinct from epithelial cells (Figure 1c). Separate sections showed strong staining of these cells with chromogranin 362 363 A, suggesting that they were primarily enterochromaffin cells which lie interspersed within the 364 colorectal epithelia (Figure 1d).

365

366 Drug transporter gene expression in colorectal and rectal cell lines

In the two colorectal cell lines, Caco-2 and HT-29, mRNA expression could be detected in 53 367 and 36 of the 84 drug transporters studied, respectively. SLC10A1/NTCP (RQ 66.5± 29.2) 368 was the most highly expressed uptake transporter followed by efflux transporter 369 ABCC2/MRP2 (RQ 12.7±0.1) in Caco-2 cells whereas, SLC7A11 (RQ 8.4±2.9) was the most 370 371 highly expressed uptake transporter and ABCC3/MRP3 (RQ 5.0±0.1) the highest expressed efflux transporter in HT-29 cells. There was no mRNA expression of ABCB1/P-gp noted in 372 373 HT-29 cells. The drug transporter expression profile of all cell lines tested is detailed in Tables 1 and 2 and Supplementary Table 1. In SW1463 rectal cell line the highest expressed drug 374 transporters were ABCB1/P-gp, SLC29A2/ENT2, SLC28A3/CNT3, SLCO4A1/OATPE, 375 SLC38A5, SLC2A1/GLUT1, SLC7A5/LAT1 and ABCC3/MRP3 (RQ 32.4± 16.9, 19.4± 6.7, 376 11.8± 1.5, 11.7±1.6, 9.8± 3.0, 8.1± 4.3, 4.1± 2.2 and 4.1± 2.2 respectively). In SW837, HRA-377 16 and HRT-18 rectal cell lines the highest expressed drug transporters were 378 SLCO4A1/OATPE (RQ 12.8±7.4, 6.2± 2.3 and 3.1±0.1) SLC38A5 (RQ 3.2 ±1.9, 12.7± 1.5) 379 and 0.01± 0.0) and SLCO1B3/OATP8 (RQ 3.2± 0.9, 3.1± 0.4 and 12.6± 0.1) respectively 380 (Tables 1 and 2 and Supplementary Table 1). 381

- 382
- 383 Comparison of relative mRNA expression between colorectal biopsies and cell lines

384 Relative gene expression of ABC and SLC transporters in colorectal tissue, colonic cell lines and rectal cell lines are summarised in Tables 1 and 2. Variable correlation between colorectal 385 tissues and cell lines was observed as determined by Pearson's correlation (r = 0.64-0.84) 386 with r values of 0.67-0.77 for Caco-2 and 0.71-0.75 for HT-29. To give an overview of the 387 388 mRNA expression profile of all the 84 target genes in the samples tested, hierarchical cluster analysis was performed. Analysis indicated that samples fell into 3 distinct clusters. Based on 389 gene expression levels three major clusters were observed. The colorectal biopsies and the 390 391 Caco-2 cell line clustered separately whereas the third cluster incorporated all the other cell 392 lines (Figure2). The distinct patterns of gene expression between the colorectal biopsy samples and the colorectal cell line were elucidated by PCA (Figure 3). The relative 393 394 contribution of the Δ Ct variance was shown by two major principal components PC1 and PC2, plotted in two dimensions of the scatterplot. The PC1 explained 38% of the variation and was 395 396 mainly dependent on the expression of ABCG2/BCRP, ABCC3/MRP3, ABCD3, SLC29A2/ENT2, SLC22A1/GLUT1 and SLC25A13/ CITRIN drug transporter genes. The PC2 397 398 explained 18.7% of the variation and was mainly dependent on the expression of SLC2A3, SLC7A5, TAP1 and ABCA4. The recto-sigmoid and rectum biopsies were in the same cluster 399 400 confirming no differences between these two anatomical sites. The human colorectal biopsies, CaCo-2 cells, HT-29 cells and the rectal cell lines were distinguishable. The colorectal biopsy 401 402 samples clustered closest with the Caco-2 cell line with the least degree of variance. The HT-29 cell line clustered separately from the biopsy samples. All the rectal cell lines were clustered 403 together but separate from the colorectal biopsies. 404

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406 **ARV drug concentrations for stimulation assays**

The viability of cell lines was assessed using Alamar Blue cell viability assays to determine the optimum drug concentrations to be used for stimulation studies. The drug concentrations which showed inhibition of cell proliferation not greater than 20% were used to stimulate the cell lines. Tenofovir showed no reduction in viability at concentrations up to 5mM, whereas

- darunavir and dapivirine showed cell vitality within acceptable limits (90±10%), at
 concentrations of 10 µM and 250 µM respectively.
- 413

414 Stimulation of Caco-2 cells with ARV drugs

415 Since Caco-2 cells had the closest match with the drug transporter profile in colorectal tissues based on PCA and also due to the presence of P-gp expression, which was absent in HT-29 416 cells, we tested the effect of ARV drugs on this cell line. Stimulation of Caco-2 cells with 417 418 tenofovir for 72 hours resulted in up-regulation of several drug transporters. Around two-fold 419 inductions were noted for LAT2, GLUT1, MVP, MRP5, OATPE and SLC38A5. However this 420 difference did not reach statistical significance for OATPE and SLC38A5. Conversely, around 421 two-fold down-regulation was seen with OCT3, VDAC2, OAT4, PEPT2, IBAT, BAT1, ABCA1, NTCP, MRP4 and AQP1 respectively. The change was statistically significant for PEPT2, 422 423 BAT1, ABCA1 and AQP1 (Figure 4a). Darunavir stimulation for 72 hours led to a two-fold increase in mRNA expression of GLUT1, PEPT1 and ABCA1 and a two-fold down-regulation 424 of SLC38A2, MRP1 and PEPT2 with the latter two differences being statistically significant 425 (Figure 4b). Dapivirine led to the positive stimulation of several transporters after 72 hours 426 427 incubation (Figure 4c) but the only significant difference was noted with SLC3A2. Similarly ABCA1 was the only drug transporter that showed statistically significant down-regulation of 428 mRNA (Figure 4c). No expression changes were observed in cells stimulated for 24 hours. 429 Stimulation periods of 168 hours (7 days) resulted in expression changes comparable to those 430 seen after 72 hours. Stimulation with the higher dose of tenofovir film (~2 mg) led to around 431 two fold significant up-regulation of MRP5, OATPE and LAT2 mRNA expression and down-432 regulation of ENT2 and MRP3 (1.5 fold) mRNA and a similar trend was seen for the lower 433 dose of tenofovir film (~1 mg) (Figure 5a). Stimulation of Caco-2 cells with darunavir film (~0.28) 434 mg) induced significant up-regulation of BCRP (1.8 fold) and OATPE (1.6 fold) genes (Figure 435 5b). 436

- 437
- 438 Explant culture

439 To investigate the suitability of the colorectal explant model for assessment of the effect of ARV drugs on drug transporter gene expression in human tissue, the histology of biopsy 440 samples was assessed with H&E staining after 24 hr incubation at different experimental 441 conditions. Out of the eight pieces of biopsies collected from an individual subject (EU56), a 442 443 pair of biopsies were immediately formalin fixed on arrival in the lab to act as controls (Figure 444 6a), two biopsy samples were incubated in media (Figure 6b), two with media containing 1% DMSO vehicle (Figure 6c) and two pieces with one of the ARVs (10µM DPV) (Figure 6d) for 445 446 24 hr on gel foam rafts. There was a uniform loss of crypt architecture with loss of epithelial 447 cells observed after 24 hr but no obvious changes in the lamina propria of the explant (Figure 6b-d). Baseline drug transporter gene expression profile of colorectal explants was studied (4 448 449 healthy subjects) in explants incubated for 24hr incubation in medium alone. The quality of the RNA extracted (RNA Integrity Number (RIN) = 7.28 ± 0.2) was comparable to RIN values 450 451 obtained for RNA extracted from colorectal biopsies immediately snap frozen after collection (RIN = 7.24 ± 0.6). Expression of most drug transporters was comparable to the expression 452 seen in fresh biopsy samples with high expression of CNT2, P-gp and MRP3 (Tables 1 and 2, 453 supplementary Table 1). However, after 24 hr incubation there was a selective loss or 454 455 downregulation of expression of BCRP, ENT2 and ABCD3 which were found to be highly expressed in colorectal samples processed immediately on collection (Tables 1 & 2). 456

457 **Explant stimulation with ARVs**

Biopsy samples from three subjects (EU54, EU55 and EU60) were used to study the drug transporter expression profile after incubation with the three ARV drugs. Out of the eight pieces of biopsies from each individual, a pair was used for incubation with each of the three drugs and the last pair of biopsies was incubated with control media. Induction of colorectal explants with TFV resulted in more than 2 fold up-regulations of MRP-1 and MRP-2 genes and a concomitant more than two-fold downregulation of MRP-4, LAT-2 and OATPD genes (Figure 7a). On the other hand, stimulation of explants with DPV led to >2 fold down-regulation of MRP-1 and OATPE (Figure 7b). Darunavir led to down-regulation by more than two fold of
 MRP3, CNT2 and OATPE (Figure 7c). The significant results are summarized in Figure 7.

467

468 **DISCUSSION**

469 This study describes mRNA expression profiles of 84 drug transporter genes in normal colorectal biopsies and representative cell lines and the effect on expression of exposure to 470 ARV drugs. To our knowledge, this is the first study to evaluate such an extensive set of drug 471 transporters in the colorectal epithelium and the effect by ARV drugs and will inform future 472 473 studies defining the role of drug transporters in the pharmacokinetics of topically applied ARV drugs. We found that 52 out of 84 drug transporters were consistently expressed in the 474 colorectal biopsy samples confirming organ-specific drug transporter expression profiles in the 475 colon.^{20, 21} The data from colorectal biopsies showed that gene expression of drug transporters 476 477 was not affected by age or gender and showed very little intra and inter-individual differences. In a previous study, age-related changes of drug transporter gene expression was noted when 478 comparing intestinal biopsy samples from paediatric and adult subjects.³⁴ We did not find any 479 demonstrable differences of gene expression of drug transporters among the adult subjects 480 481 screened in this study. Gender related differences in drug transporters have not been specifically studied in the colon. In the analyses presented here we show no difference of gene 482 expression of drug transporters between males and females. Previous reports have suggested 483 that P-gp expression is lower in peripheral mononuclear cells of female subjects while BCRP 484 expression was equal in the two sexes.^{35, 36} There was no distinction between the gene 485 486 expression in biopsy samples taken from the rectum or the contiguous distal sigmoid colon. Studies on delivery of rectal microbicides indicates the extent of reach of these formulations 487 is about 60 cm from the anal verge.³⁷ The lack of difference of drug transporter gene 488 expression between the rectum and the recto-sigmoid would suggest a more predictable, 489 uniform uptake of putative microbicidal drugs from the distal recto-sigmoid segment. We show 490

high expression of the ABC transporters, P-gp, BCRP and MRP3 in colorectal biopsies which 491 is consistent with findings from previous studies.^{20-22, 24, 38, 39} The mRNA expression of P-gp 492 was confirmed by immunohistochemistry which showed uniform staining of the microvilli. High 493 expression of P-gp in the colon will have a significant impact on ARV drugs being considered 494 495 as preventive rectal microbicides. P-gp acts as a gatekeeper for several xenobiotics and directly determines their bioavailability.⁴⁰ It has been implicated in efflux of NRTIs like tenofovir 496 and abacavir but was shown to be inhibited by PIs and NNRTIS.⁴¹⁻⁴⁴ This suggests that a drug 497 combination of NRTIs and PIs or NNRTIs may impact P-gp in opposing fashions and be 498 mutually beneficial in maintaining tissue concentrations. This theoretical benefit was not 499 demonstrable in a volunteer study which showed increased P-gp expression in ARV-treated 500 patients compared to ARV-naïve HIV subjects.⁴⁵ The interpretation of these findings is difficult 501 502 as the 16 patients recruited were on 9 different drug combinations. The study also 503 demonstrated that HIV-1 infection in itself down-regulated P-gp expression when biopsies from ARV-naïve HIV-1 patients were compared to uninfected individuals. In our study BCRP 504 505 was also found to be highly expressed in the colorectal biopsies. Similar to P-gp, BCRP also 506 mediates efflux of NRTIs and is inhibited by PIs and NNRTIs and as a result shares similar clinical implications.46,47 507

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509 MRP3 showed high expression in colorectal tissue in our study in agreement with other reports.^{20, 24, 38, 39} MRP3 is highly expressed in the liver and intestine and is involved in the 510 physiological regulation of bile salt enterohepatic circulation.⁴⁸ The basolateral localization of 511 MRP3 in the enterocyte may facilitate transfer of drugs to the portal circulation.⁴⁹ A study based 512 on transfected cell lines suggested that NRTIs and NNRTIs can inhibit MRP3.⁵⁰ Our findings 513 of low expression of MRP1, MRP4, MRP5 and MRP6 and no expression of MRP2 are 514 consistent with previous reports.^{21, 22, 39} One of these studies showed protein but not mRNA 515 expression of MRP2 in cadaveric colonic samples.²⁴ Clinical and in vivo data have 516 demonstrated that tenofovir is a substrate for MRP4 while the role for MRP2 is less clear. ^{51,} 517 518 ⁵² The lack of expression of MRP2 and MRP4 in the colon may have important implications

for tenofovir-based microbicidal formulations, namely greater retention of tenofovir in 519 colorectal tissue as demonstrated in a healthy volunteer study measuring drug penetration 520 after an oral dose of the drug.⁵³ In this study rectal concentrations of tenofovir were found to 521 be 100-fold higher than that in the vagina and could be partially explained by expression of 522 MRP2 in the vagina.^{54, 55} We have also documented increased expression of ABCD3, which 523 is a peroxisomal transporter that is involved in the transport of long and branched chain acyl-524 CoA and bile acid intermediates and has not been reported to have any interaction with ARV 525 drugs.⁵⁶ 526

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Uptake transporters CNT2, CNT3, ENT2 and MCT1 were found highly expressed in colorectal 528 biopsy samples, consistent with a study which specifically assessed these transporters.²⁰ 529 530 CNT2, CNT3 and MCT1 are nucleoside transporters involved in the transport of hydrophilic nucleosides and nucleoside analogs within cells.⁵⁷ In this study we provide detailed 531 information on localisation of ENT2 and CNT2 protein utilising immunohistochemistry. The 532 staining for ENT2 showed uniform nuclear staining of all epithelial cells, but CNT2 staining 533 was concentrated on few cells interspersed within the epithelium. CNT2 positive cells stained 534 535 with chromogranin suggesting they were enterochromaffin cells. A further study also found significant expression of these two transporters but did not find any detectable expression of 536 CNT3 expression.⁵⁸ Nucleoside transporters, more specifically CNT2 and CNT3 are involved 537 in the uptake of NRTI's and PI's and their increased expression will enhance the bioavailability 538 of these drugs.^{59, 60} The other uptake transporter which showed a high expression was MCT1 539 which is a ubiquitous transporter detected in the intestine and colon.^{61, 62} Importantly, our study 540 did not show significant expression of any of the OAT or OCT group of drug transporters as 541 previously shown.^{21, 22, 63} However, other studies have recorded low mRNA expression of 542 OAT1, OCT3 in colorectal biopsies with one study demonstrating immunohistochemical 543 staining of OAT1 in scattered epithelial cells.^{38, 63} We have also found low expression of 544 OATP2B1 in agreement with other studies²⁴, which is likely to impact distribution of rectally-545 546 applied PIs as it is a substrate for PIs and is potently inhibited by some of them.¹⁸

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548 Gene expression profiles of colorectal biopsies were compared to six colonic and rectal cell lines to confirm the suitability of these organotypic cell lines as surrogates for human colorectal 549 tissue. Caco-2 cells, which originate from a colonic adenocarcinoma cell line has been the 550 551 most widely reported model to study carrier-mediated uptake and efflux mechanisms with full differentiation reported after approximately 21 days. The data reported in the present study 552 are comparable to the collated results of the drug transporter gene expression of Caco-2 cells 553 from 10 different laboratories.²⁸ In another study, gene expression of the HT29 cell line was 554 555 reported to be the closest match with human colorectal samples when considering 377 genes 556 encompassing not only drug transporters but also xenobiotic metabolizing enzymes, nuclear receptors and transcription factors but data described here do not support this finding.²⁰ We 557 have noted differences in the mRNA profile in HT-29 and Caco-2, with enhanced expression 558 559 of MRP2 in Caco-2 cells and lack of expression of P-gp in the HT29 cells in comparison to the colorectal tissue samples (Table 1 and 2). Specific focus on the 84 drug transporter gene 560 expression with hierarchical clustering and PCA analysis in this study showed that Caco-2 561 clustered separately but had the closest relation with colorectal biopsies compared to the other 562 563 cell lines (Figure 1 and 3). To our knowledge this is the first report of drug transporter expression in rectal cell lines SW1463, SW837, HRA-16 and HRT-18. P-gp and MRP3 were 564 expressed similarly but BCRP was much lower than the colorectal biopsy samples with wide 565 variations in the expression of SLC transporters. This was further confirmed by the principal 566 component analysis which demonstrated a high degree of variance of the rectal cell lines with 567 colorectal biopsies. This study shows that the Caco-2 cell line was more a suitable surrogate 568 model compared to rectal cell lines to study drug transporter gene expression in human 569 colorectal tissue, although we acknowledge reports indicating that expression of transporters 570 in Caco-2 cells was closest to that seen in the small intestine.²¹ 571

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573 In light of these findings Caco-2 cells were chosen for induction studies with ARV drugs 574 currently in the pipeline of microbicides development, namely tenofovir, dapivirine and

575 darunavir. The drug concentrations for stimulation studies were determined keeping in mind the potential cytotoxic effects on the cell lines and data from pre-clinical and clinical studies. 576 A recent phase 1 study has confirmed safety of 1% tenofovir gel as rectal microbicide.⁶⁴ 577 Rectally-applied tenofovir gel showed protection in NHP studies at a concentration of 1% and 578 579 the concentration of tenofovir retrieved in rectal fluid of macaques was in the range of 9 mM, which was higher than drug concentrations used to stimulate the Caco-2 cell lines in our 580 study.⁶⁵ The NNRTI dapivirine has been incorporated in a vaginal ring and is currently being 581 tested in phase 3 clinical trials but this has not been utilised for rectal delivery.⁶⁶ A 582 583 dapivirine/darunavir combination vaginal ring has also undergone pre-clinical trial in a macaque model and this combination is being tested as a vaginal microbicide in the DAPIDAR 584 585 trial.⁶⁷⁻⁶⁹ No pre-clinical or clinical studies are available for rectal formulations of this 586 combination. Nonetheless, the concentrations of dapivirine and darunavir used for stimulation 587 of cell lines in this study were determined by results of the cytotoxicity assays and concentrations that maintained solubility in culture medium. 588

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Stimulation of Caco-2 cell lines with ARV drugs showed altered mRNA expression of key 590 591 genes for ARV drug transporters. Darunavir significantly down-regulated MRP1 implicated in transport of both PIs and NRTIs, some of which are also known to inhibit this transporter.¹⁸ 592 This is likely to affect net drug transport across the epithelial barrier depending on the cell 593 membrane distribution of MRP1. On the other hand, concurrent up-regulation of BCRP which 594 we detected in Caco-2 cells stimulated with formulated darunavir could counteract the effect 595 of MRP1 down-regulation. A previous report showed that darunavir increased the mRNA 596 expression of P-gp after one week of incubation of LS180 colorectal cells.⁷⁰ In our study, a 597 598 very modest level of darunavir-induced up-regulation of P-gp was observed in Caco-2 cells 599 stimulated with formulated drug only and did not reach statistical significance. Formulated 600 darunavir decreased gene expression of MRP4, albeit at non-statistically significant levels. 601 MRP4 is mostly expressed on the apical cell membrane and is responsible for rapid efflux and 602 reduced bioavailability of tenofovir.⁵¹ Tenofovir in both forms as well as formulated darunavir

603 also up-regulated expression of the mostly baso-lateral MRP5 transporter. MRP4 downregulation and MRP5 up-regulation may facilitate distribution of tenofovir to sub-epithelial 604 target cells. On the other hand, MRP5 has been considered to be a factor for unexplained 605 drug resistance in HIV-1 patients.⁷¹ Tenofovir in the formulated form was also found to 606 607 increase expression of OATPE, a member of the SLCO transporter family. SLCO transporters have been implicated in transport of PIs¹⁸ and as such tenofovir may increase intracellular 608 accumulation of darunavir if combined with this drug, thus decreasing distribution to sub-609 610 epithelial CD4+ T cells. Dapivirine showed no effect on expression of key transporters involved 611 in efflux and influx of nucleotide analogues. Up-regulation of OATPE and down-regulation of 612 OATP2B1 by dapivirine may affect net transport of darunavir but expression changes of these 613 transporters did not reach statistical significance. There were divergent effects of tenofovir, 614 darunavir and dapivirine on the expression of the ABCA1 transporter which is a cholesterol efflux regulatory protein known to be down regulated during HIV-1 replication.⁷² Our findings 615 suggest that darunavir may have the potential to reverse this change by up-regulation of 616 ABCA1, the implication of which is yet to be determined. Keeping in mind the primary objective 617 of utilising these drugs as constituents of a rectal microbicide, the induction studies with 618 619 tenofovir, darunavir and dapivirine have not shown expression changes of key efflux or uptake transporters that negatively impact their individual distribution to sub-epithelial target cells. In 620 light of the effect of darunavir against MRP transporters known to be substrates for tenofovir, 621 distribution of tenofovir to target cells in the colorectal region could be enhanced by 622 combination with darunavir. On the other hand the effect of tenofovir on SLCO transporters 623 may negatively impact on darunavir distribution to sub-epithelial target cells. These findings 624 will inform detailed pharmacokinetic modelling studies and pre-clinical studies of these 625 microbicide combinations in non-human primates.⁷³ The induction and expression of drug 626 transporters in the colorectal epithelium will determine pharmacokinetics of microbicides from 627 the luminal compartment to the submucosal compartment as they may influence drug 628 absorption and distribution. 629

630

631 Colorectal explant culture systems have been used as a surrogate model to evaluate safety and efficacy of topical microbicides for the prevention of HIV infection.^{9, 14} We performed a 632 preliminary study to look at the effectiveness of this model to assess changes in drug 633 transporter expression after induction with different ARV drugs. Histologically there was 634 635 shedding of the crypt epithelium even after 24 hours in the explant samples cultured with 636 medium alone. We observed a selective loss of expression of key drug transporters like BCRP and ENT2 after this period. Nonetheless, good guality mRNA could be extracted from the 637 638 explants after 24 hours and showed expression of a majority of drug transporters including 639 CNT2, P-gp and MRP3 similar to that seen in fresh biopsy samples. The tissue explant model is a valuable tool for pre-clinical assessment of inhibition of HIV-1 infection by topical 640 641 microbicides acting on mucosal CD4+ T cells but may be less than ideal for studying the effect 642 of ARV stimulation on drug transporter expression in the explant epithelium.¹⁴ There was a 643 selective loss or downregulation of expression of certain important genes like BCRP, ENT2 and ABCD3 after 24 hours in the explant model with a parallel histological loss of the colorectal 644 645 epithelium. In this background, stimulation data after incubation with ARV drugs may be unreliable. This is exemplified to a certain extent by comparison of the ARV induced gene 646 647 expression data in Caco-2 cells and the explant culture system. For instance, stimulation of Caco-2 cells with Tenofovir and Dapivirine upregulated OATPE gene expression whereas the 648 exact converse was seen in the explant culture model. Time-course studies with a larger 649 number of samples will be needed to optimise this model to study ARV stimulation. 650

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This is the first study to provide an extensive assessment of the expression of all drug transporters in the rectum and distal colon that are likely to be involved in the pharmacokinetics of rectally-delivered ARV-based microbicides. High expression of ABC transporters P-gp, BCRP and MRP3 and the SLC transporters CNT2, CNT3, ENT2 and MCT1 in normal colorectal biopsies observed in this study needs to be considered in design of microbicide formulations. This study shows that Caco-2 is the most appropriate surrogate cell line to pursue *in vitro* transport kinetics studies of candidate microbicides. Induction of Caco-2 cell

659 lines with darunavir and tenofovir suggests that darunavir-based microbicides incorporating tenofovir may result in drug-drug interactions likely to affect distribution of individual drugs. 660 The findings from this study will inform development of microbicidal combinations that will be 661 both effective and safe for pre-exposure prophylaxis against HIV-1 infection. Our study aimed 662 663 to elucidate drug transporter gene expression in the colorectal epithelium which is a factor determining drug delivery to the target cells, namely the CD4+ T cells in the lamina propria. 664 The findings of the effect of ARVs on tissue explants provides an indication of the overall effect 665 666 on drug transporter expression in all cell types of the colorectal mucosa. However, data may 667 be confounded by the loss of histological integrity observed after 24h culture. 668 669 Pharmacokinetics studies in non-human primate models and ultimately within clinical trials will

elucidate the linkage of drug transporter expression in the epithelium and mucosal
concentrations of ARV. The number of CD4+ T cells that we could retrieve in the endoscopic
biopsy samples was too small to study the concomitant expression changes in CD4+ T cells.
Future studies will establish drug transporter expression in submucosal CD4+ T cells and the

- 674 pharmacokinetics of ARV microbicides within the target CD4+ T cells.
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682

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	Common Name	Colorectal Tissue, <mark>n=12*</mark>	Colorectal cell line		Rectal cell line				Colorectal
Gene			Caco-2	HT-29	SW1463	SW837	HRA16	HRT18	Explant 24 hr, n=4
ABCA1	ABCA1	+	+	-	-	-	-	-	++
ABCA12	ABCA12	+/-	-	-	-	+	+	++	+++
ABCA13	ABCA13	+/-	-	-	-	-	-	-	-
ABCA2	ABCA2	+	+	+	+	+	+	+	-
ABCA3	ABCA3	-	-	-	-	+	-	-	-
ABCA4	ABCA4	-	-	-	-	-	-	-	-
ABCA9	ABCA9	+	-	-	-	-	-	-	-
ABCB1	Рдр	+++	+++	-	+++	+	+++	++	+++
ABCB11	BSEP	+	-	-	-	-	-	-	+
ABCB4	MDR3	+	-	-	-	-	-	-	+
ABCB5	ABCB5	-	-	-	-	-	-	-	-
ABCB6	ABCB6	+	++	+	+	+	+	+	+
ABCC1	MRP1	+	++	+	++	++	+	+	+
ABCC10	MRP10	+	+	+	++	+	+	+	+
ABCC11	MRP8	-	-	-	-	-	-	-	-
ABCC12	MRP9	-	-	-	-	-	-	-	-
ABCC2	MRP2	-	+++	-	+++	-	+++	-	-
ABCC3	MRP3	+++	+++	+++	+++	+++	++	+	++
ABCC4	MRP4	+	++	++	++	+	+	+	++
ABCC5	MRP5	+	+	-	++	+	+	+	+
ABCC6	MRP6	+	+	-	+	-	+	-	-
ABCD1	ABCD1	+	-	+	+	++	-	+	+
ABCD3	ABCD3	+++	+	+	++	+	+	+	+
ABCD4	ABCD4	+	+	+	+	+	+	+	+
ABCF1	ABCF1	+	+	+	++	++	+	++	++
ABCG2	BCRP	+++	++	+	+	+	-	-	-
ABCG8	ABCG8	+/-	-	-	-	-	-	-	-
AQP1	AQP1	-	-	-	-	-	-	-	-
AQP7	AQP7	-	-	-	-	-	-	-	-
AQP9	AQP9	-	-	-	-	-	-	-	++
ATP6V0C	ATP6V0C	+	+	+	++	+	+	+	+
ATP7A	ATP7A	+	+	+	+	+	+	+	+
ATP7B	ATP7B	+	+	-	++	-	+	-	+
MVP	MVP	++	+	-	++	+	+	+	+++
TAP1	TAP1	+	-	-	+	+	+	+	+++
TAP2	TAP2	+	+	+	+	+	+	+	++
VDAC1	VDAC1	++	+	+	++	+	+	+	+
VDAC2	VDAC2	++	+	+	+	+	-	-	+

Table 1: Expression of ABC transporters in colorectal tissues and representative cell lines

Rectal and recto-sigmoid biopsies, Genes in bold are implicated in ARV drug transport, RQ=Relative quantification.

High expression, Mean RQ >2

Moderate expression, Mean RQ 1-2

Low expression, <mark>Mean RQ</mark> <1

Unexpressed, +/-Mean RQ < 0.1

Variable expression (Observed in 33% -92% subjects)

+

	Common Name	Colorectal Tissue, <mark>n=12*</mark>	Colorectal cell		De stal es ll line				Colorectal
Gene			line		Rectal cell line				Explant, 24
			Caco-2	HT-29	SW1463	SW837	HRA16	HRT18	<mark>hr, n=4</mark>
SLC10A1	NTCP	+/-	+++	-	-	-	-	-	-
SLC10A2	IBAT	+/-	+	-	-	-	-	-	-
SLC15A1	PEPT1	+	++	-	+++	-	++	-	++
SLC15A2	PEPT2	+	+	-	-	-	-	-	-
SLC16A1	MCT1	+++	+	+	+++	+	+	+	+
SLC16A2	MCT8	+/-	-	-	-	-	-	-	-
SLC16A3	MCT3	++	+	++	++	+++	+	++	+++
SLC19A1	SLC19A1	+	+	+	+++	+++	++	+++	+
SLC19A2	THTR1	+	++	+	+	+	+	+	+
SLC19A3	THTR2	+	+++	-	+	-	+	-	-
SLC22A1	OCT1	-	+	-	+	-	+	-	+/-
SLC22A2	OCT2	-	-	-	-	-	-	-	-
SLC22A3	ОСТЗ	-	-	+	+++	+	+	-	+
SLC22A6	OAT1	-	-	-	-	-	-	-	-
SLC22A7	OAT2	-	-	-	-	-	-	-	-
SLC22A8	OAT3	-	-	-	-	-	-	-	-
SLC22A9	OAT4	-	-	-	-	-	-	-	-
SLC25A13	CITRIN	++	++	++	+++	++	++	++	+
SLC28A1	CNT1	-	-	-	-	-	-	-	-
SLC28A2	CNT2	+++	-	-	-	-	-	-	+++
SLC28A3	CNT3	+++	+	+++	+++	-	+++	+	+++
SLC29A1	ENT1	+	++	+	+++	+	+	+	+
SLC29A2	ENT2	+++	+++	+++	+++	+++	+++	+++	-
SLC2A1	GLUT1	+	+++	+++	+++	+++	+++	-	+++
SLC2A2	GLUT2	-	+	-	-	-	-	-	-
SLC2A3	GLUT3	-	+++	-	-	+	-	+	+
SLC31A1	SLC31A1	+	+	++	++	+	+	+	+
SLC38A2	SLC38A2	++	+	++	+++	+	+	+	+
SLC38A5	SLC38A5	+	-	++	+++	+++	+++	-	++
SLC3A1	NBAT	+	+	-	+	-	-	-	-
SLC3A2	SLC3A2	+	+	++	+++	+	+	+	+
SLC5A1	SGLT1	++	+	+	+++	+	+	-	+++
SLC5A4	SGLT3	-	-	-	-	-	-	-	-
SLC7A11	SLC7A11	+	+	+++	+++	+	+	+	+++
SLC7A5	LAT1	-	+	+++	+++	+++	+	+++	+
SLC7A6	LAT3	+	+++	+	+++	+++	++	++	+
SLC7A7	YLAT-1	-	++	-	+	+	+	-	-
SLC7A8	LAT2	+	+	-	+	-	+	+	_
SI C7A9	BAT1	_	+	-	+	-	-	-	-
SLCO1A2	ΟΑΤΡ	_	+	-	-	_	_	_	_
SLCO1B1	OATPC	_	-	_	+	_	_	+	_
SLCO1B3	OATP8	_	-	+++	+	+++	+++	+++	_
SI CO2A1	ΟΔΤΡ2Δ1	+	-	_	-	+	_	_	+
SICO2R1	ΟΔΤΡ2R1	, +	+++		_				-
SICO2D1		+			_	+	+		+
SLCO4A1	OATPE	+	+	++	+++	+++	+++	+++	+++

Table 2: Expression of SLC transporters in colorectal tissues and representative cell lines

Rectal and recto-sigmoid biopsies, Genes in bold are implicated in ARV drug transport, RQ=Relative quantification.



Moderate expression, Mean RQ 1-2 Low expression, Mean RQ <1

n, -L Unexpressed,

Mean RQ < 0.1

+/- Variable expression (Observed in 33% -92% subjects)

+

Figure 1: Immunochemical detection of drug transporters in colorectal tissue. Representative photomicrographs of (A) Colorectal tissue stained with monoclonal antibody JSB-1 against P-gp; (B) Colorectal tissue stained with monoclonal antibody EPR1164 against ENT2; (C) Colorectal tissue stained with polyclonal antibody ab79993 against CNT2; (D) Colorectal tissue stained with monoclonal antibody M0869 against chromogranin A. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

(a) ABCB1/P-gp



(c) SLC28A2/CNT2

(b) SLC29A2/ENT2



(d) Chromogranin A





Figure 2: Hierarchical clustering of mRNA expression of drug transporters in colorectal biopsies and six cell-lines. Distances between samples and assays are calculated for hierarchical clustering based on the ΔCT values using Pearson's correlation. Normalization was done using three endogenous control genes (HPRT1, PGK1 and PPIA) and the ΔCT values were calculated by subtracting the mean of the CT values of the endogenous controls from the CT value of the gene for each sample. The rows of the heat map represent 84 drug transporter genes and the columns represent samples. The ΔCT value of the neutral/middle expression level (mean) is set such that red indicates an increase with a Δ CT value below the middle level, and green indicates a decrease, with a ΔCT value above the middle level. The relationship between colours and normalized values of gene expression is illustrated in the key at the bottom of the figure. Three major clusters were identified in the dendrogram based on gene expression levels. The colorectal biopsies from two sites (EU3 to EU10, EU12, EU13, EU14 and EU16) and the Caco-2 cell line clustered separately whereas the third cluster incorporated all the other cell lines (HT-29, SW1463, SW837, HRT-18 and HRA-16) suggesting closer linkage of Caco-2 cells to colorectal tissue drug transporter gene expression. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.



Figure 3: Principal component analysis of gene expression profiles generated from colorectal biopsy samples and six cell lines. Relative contribution of the Δ Ct variance is shown by two major principal components (PC1 and PC2) plotted in two dimensions. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.



Figure 4: Effect of antiretroviral drugs on mRNA expression of drug transporters in Caco-2 cells. Mean RQ of genes up and down regulated in Caco-2 cells induced with (a) 5mM tenofovir (b) 250µM darunavir and (c) 10µM dapivirine for 3 days compared with appropriate medium controls. *P<0.05; **P<0.01 and ***P<0.001. The drug concentrations used were found to be non-toxic to the Cao-2 cells. The genes which were up and down regulated more than 1.5 times have been included in this figure.



Figure 5: Effect of tenofovir and darunavir dissolving films on mRNA expression of drug transporters in Caco-2 cells. Mean RQ of genes up and down regulated in Caco-2 cells induced with (a) tenofovir films and (b) darunavir films for 3 days compared with appropriate respective placebo films as controls. *P<0.05; **P<0.01 and ***P<0.001.



(a) Tenofovir

Figure 6: Explant histology. Haematoxylin and eosin staining of formalin fixed paraffin embedded colorectal explant. Panel (a) shows staining of colorectal biopsy samples immediately formalin fixed on arrival in the lab, panel (b) shows biopsy samples incubated in media for 24 hr on gel foam rafts, panel (c) shows staining of colorectal biopsy samples incubated in media with 1% DMSO for 24 hr on gel foam rafts and panel (d) shows staining of colorectal biopsy samples incubated in media with ARV (10µM DPV) for 24 hr on gel foam rafts. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

(a) Normal colorectal biopsy, 1 hr after collection

(b) 24 hr explant culture in media



(c) 24 hr explant culture in media with 1% DMSO



(d) 24 hr explant culture in media with ARV (10µM DPV)





Figure 7: Effect of antiretroviral drugs on mRNA expression of drug transporters in colorectal explants. Mean RQ of genes up and down regulated in colorectal explants induced with (a) 5mM tenofovir (b) 250µM darunavir and (c) 10µM dapivirine for 24hr compared with appropriate medium controls. *P<0.05; **P<0.01 and ***P<0.001.

(b) Dapivirine



(a) Tenofovir



