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Murphy, D. J., Desjardins, D., Dereuddre-Bosquet, N., Brochard, P., Perrot, L., Pruvost, A., ... Malcolm, R. K. (2014). Pre-clinical development of a combination microbicide vaginal ring containing dapivirine and darunavir. DOI: 10.1093/jac/dku160

Document Version:
Publisher's PDF, also known as Version of record

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Journal of Antimicrobial Chemotherapy

Pre-clinical development of a combination microbicide vaginal ring containing dapivirine and darunavir

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Objectives: Combination microbicide vaginal rings may be more effective than single microbicide rings at reducing/preventing sexual transmission of HIV. Here, we report the pre-clinical development and macaque pharmacokinetics of matrix-type silicone elastomer vaginal rings containing dapivirine and darunavir.

Methods: Macaque rings containing 25 mg dapivirine, 100 mg dapivirine, 300 mg darunavir or 100 mg dapivirine+300 mg darunavir were manufactured and characterized by differential scanning calorimetry. *In vitro* release was assessed into isopropanol/water and simulated vaginal fluid. Macaque vaginal fluid and blood serum concentrations for both antiretrovirals were measured during 28 day ring use. Tissue levels were measured on day 28. *Ex vivo* challenge studies were performed on vaginal fluid samples and IC_{50} values were calculated.

Results: Darunavir caused a concentration-dependent reduction in the dapivirine melting temperature in both solid drug mixes and in the combination ring. *In vitro* release from rings was dependent on drug loading, the number of drugs present and the release medium. In macaques, serum concentrations of both microbicides were maintained between 10^1 and 10^2 pg/mL. Vaginal fluid levels ranged between 10^3 and 10^4 ng/g and between 10^4 and 10^5 ng/g for dapivirine and darunavir, respectively. Both dapivirine and darunavir showed very similar concentrations in each tissue type; the range of drug tissue concentrations followed the general rank order: vagina (1.8×10^3–3.8×10^3 ng/g) >cervix (9.4×10^1–3.9×10^2 ng/g) >uterus (0–108 ng/g) >rectum (0–40 ng/g). Measured IC_{50} values were >2 ng/mL for both compounds.

Conclusions: Based on these results, and in light of recent clinical progress of the 25 mg dapivirine ring, a combination vaginal ring containing dapivirine and darunavir is a viable second-generation HIV microbicide candidate.

Keywords: HIV microbicides, silicone elastomer vaginal rings, cynomolgus macaques, pharmacokinetics

Introduction

Over the past 20 years, various vaginally administered products have been evaluated as topical microbicides for reduction or prevention of sexual transmission of HIV type 1 (HIV-1), although none has yet reached market. More recently, owing to lack of efficacy, focus has shifted away from microbicide compounds with non-specific mechanisms of action and towards more potent antiretroviral (ARV) drugs, similar to those used in highly active antiretroviral therapy (HAART) and targeted at specific steps in the HIV replication cycle. Various lead candidate microbicide products containing a single ARV drug are now progressing through clinical development, including a dapivirine-releasing vaginal ring,[1–3] a tenofovir-releasing vaginal ring[4] and an aqueous tenofovir gel.[5] Following the combinatorial drug approach established with HAART,[6] next-generation microbicides will also likely be combination drug products or multipurpose prevention technologies, containing multiple ARV drugs or a combination of ARV, contraceptive and/or antimicrobial agents, respectively.[7] There are good reasons for pursuing combination microbicides. Compared with a single ARV drug, they may offer: (i) increased breadth of activity against emerging resistant viral strains;

Table 1. Combination HIV microbicide approaches reported in the literature

Microbicide combination	Formulation	Reference
Carrageenan+MIV-150	gel	32
Dapivirine+maraviroc	ring	11
Dapivirine+tenofovir	ring	33
Tenofovir+IQP-0528	gel	34,35
Tenofovir+UC781	gel	36
Stampidine+PHI-443	gel	37

(ii) lower clinical concentrations for efficacy due to additive or synergistic effects; and (iii) greater protection through targeting of multiple stages in the virus replication cycle.[8] To date, only a limited number of combination ARV microbicide strategies have been reported, including three vaginal ring products[9–11] and several gel formulations[12,13] (Table 1).

Here, we report the pre-clinical development of a silicone elastomer matrix-type vaginal ring containing a combination of dapivirine and darunavir ethanolate (also known as TMC114; hereafter referred to simply as darunavir). Dapivirine is an experimental non-nucleoside reverse transcriptase inhibitor (NNRTI) currently undergoing Phase III clinical testing in Africa as an HIV microbicide in the format of a 25 mg dapivirine-loaded silicone elastomer matrix-type vaginal ring.[1–3] Darunavir is a second-generation protease inhibitor used in combination with other ARVs in the treatment of HIV infection. Protease inhibitors inhibit the HIV protease enzyme required to produce mature infectious virus particles by cleaving structural proteins and enzymes from their precursors. Their high potency within HAART regimens and the relatively high genetic barrier to the emergence of resistant HIV strains (compared with other ARVs) suggest they have good potential as microbicides, administered alone or in combination with other ARVs.[14,15]

Materials and methods

Materials

Medical grade, addition-cure, silicone elastomer two-part kit (LSR9-9509-30; also known as DDU-4320) was supplied by Nusil Silicone Technology Inc. (Carpinteria, CA, USA). Darunavir and dapivirine were kindly provided by Janssen Research and Development (Beerse, Belgium) and the International Partnership for Microbicides (IPM; Silver Spring, MD, USA), respectively. HPLC-grade acetonitrile, isopropanol (IPA), methanol, dichloromethane, trifluoroacetic acid (TFA) and 19-norethindrone were obtained from Sigma–Aldrich (Gillingham, UK). Simulated vaginal fluid (SVF; pH 4.2) was prepared using analytical grade reagents according to a method described previously.[16] Deionized water, resistivity >18 mΩ/cm², was obtained from a Millipore Direct-Q3 UV Ultrapure water system (Watford, UK).

Ring manufacture

Macaque-sized, matrix-type, silicone elastomer vaginal rings (25 mm outer diameter and 6 mm cross-sectional diameter[9,17]) containing various loadings of dapivirine (25 and 100 mg), darunavir (300 mg) and their combination (100 mg dapivirine+300 mg darunavir) were manufactured by reaction injection moulding on a custom laboratory-scale ring manufacturing machine. For batch manufacture of each ring formulation, the required quantities of dapivirine and darunavir were added to both parts A and B of the silicone elastomer system in polypropylene containers. After mixing at 2000 rpm for 5 min (SpeedMixer™ DAC 150.1 FVZ-K, Synergy Devices Ltd, UK), the containers were degassed in a vacuum chamber (0.143 Torr held for 30 min; LACO Technology, Salt Lake City, UT, USA), and then stored sealed at 4°C for 60 min. Parts A and B of the silicone/drug mixture were combined in a 1:1 ratio and mixed (3000 rpm for 30 s) before being injected into pre-heated (80°C), precision-engineered, stainless steel, vaginal ring moulds and cured for 3 min. Each ring weighed ∼1.8 g.

Thermal analysis by differential scanning calorimetry (DSC)

Samples of micronized dapivirine, non-micronized darunavir and mixtures of the two drugs in various proportions were prepared for DSC analysis. Each mixture was mixed thoroughly, first by hand using a spatula and then in a Speedmixer™ at 3000 rpm. Powder samples were analysed by DSC (TA Instruments 2920 modulated DSC) in standard heating ramp mode. Approximately 5–10 mg of each sample was accurately weighed into an aluminium pan and heated from 20 to 250°C at a rate of 10°C per min alongside an empty reference pan. For each sample, the following parameters were noted for any melting transitions that were observed: onset temperature (°C), peak temperature (°C) and enthalpy (ΔH, J/g). A minimum of four replicates were used to calculate mean values for each sample mixture. DSC analysis was similarly performed on silicone elastomer samples loaded with various concentrations and ratios of dapivirine only, darunavir only and dapivirine+darunavir in order to characterize the nature of the drugs in the rings.

In vitro release testing

In vitro release of dapivirine and darunavir from the single active compound and combination rings into both IPA/water (1:1) and SVF media was assessed over a continuous 28 day period. An IPA/water mixture has been widely used for *in vitro* release testing of silicone elastomer vaginal rings containing poorly water-soluble drugs, since its greater solvating capacity offers sink conditions.[1,9,11,18] Individual rings were placed into wide-necked screw-capped 250 mL glass flasks (Duran® GLS 80®) containing 100 mL IPA/water (1:1) or SVF (n=4). The flasks were sealed and placed in a rotating orbital incubator (Infors HT Unitron, Switzerland; 37°C, 60 rpm, throw 25 mm). After 24 h (±15 min), each flask was removed from the incubator and a 2 mL sample of release medium was retained for HPLC analysis. The remaining release medium was discarded and replaced with a fresh 50 mL aliquot of either IPA/water or SVF. Sampling and release medium replacement was conducted daily except for weekends, when the release medium volume was doubled and the daily sampling routine was restarted again on the Monday. The quantities of dapivirine and darunavir released from the rings were determined using HPLC with UV detection at 257 nm. A 10 μL aliquot of each sample was injected onto a Luna 5 μm C18(2) 100 Å column (150×4.6 mm; Phenomenex, Cheshire, UK) maintained at 30°C. HPLC analysis was conducted in isocratic mode with a mobile phase comprising 0.1% TFA in water (45%) and methanol (55%) at a flow rate of 1 mL/min. Under these conditions, dapivirine and darunavir displayed retention times of 12.0 and 10.5 min, respectively. Standard solutions of dapivirine and darunavir in methanol (0.1–100 μg/mL) were used to construct a linear calibration plot for each analyte (R^2=0.9949 and 0.9929 for dapivirine and darunavir, respectively) and drug concentrations were determined for each sample. Daily (or weekend) release values were subsequently calculated and used to plot daily and cumulative release versus time.

Pharmacokinetic study in macaques

A pharmacokinetic study in adult female cynomolgus macaques (weight range 3–7 kg) was performed at Commissariat à l'Energie Atomique (CEA), IDMIT infrastructure. Non-human primates (which include *Macaca fascicularis*) are used at the CEA in accordance with French national regulations and under the supervision of national veterinary inspectors (CEA Permit Number A 92-032-02). The CEA complies with the Standards for Human Care and Use of Laboratory Animals, of the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance number #A5826-01. All experimental procedures were conducted according to European guidelines for animal care (European directive 86/609, 'Journal Officiel des Communautés Européennes', L358, December 18, 1986). The use of non-human primates at the CEA is also in conformity with the recommendations of the newly published European Directive (2010/63, recommendation No. 9). The animals were used under the supervision of the veterinarians in charge of the animal facility. This study was scientifically reviewed and approved by the EU FP7 Combined Highly Active Antiretroviral Microbicide (CHAARM) programme and was accredited under statement numbers 10-062, by the ethics committee Comité d'Ethique en Expérimentation Animale du CEA registered under number 44 by the French Ministry of Research. Four animals were used per ring treatment and there was no pre-treatment with Depo-Provera (medroxy-progesterone acetate).[9] Following atraumatic ring insertion into the vagina under anaesthesia on day 0, blood (serum) and vaginal fluid were collected at 8, 24 and 48 h and after 7, 14 and 28 days (Table 2). Blood was sampled into either EDTA tubes (for haematology) or dry tubes (for drug quantification). Vaginal fluid sampling was performed using Weck-Cel® spears (Medtronic Ophthalmics, Jacksonville, FL, USA) based on a previously described method.[2] A sample collection set containing the Weck-Cel® spear head plus a collection microtube was housed within a sterile 50 mL polypropylene tube. Each sample collection tube was pre-weighed and labelled for each animal. Sample collection sets containing the Weck-Cel® spear were stored on ice at between 2 and 8°C until sample collection. The Weck-Cel® spear was placed in the vaginal vault for 1–2 min to absorb the fluid. The spear head was then cut and placed into the collection microtube. After closure of its lid, the microtube was placed back into the corresponding outer tube. The stem of the spear was placed into the outer tube and the sample collection set was placed back on ice. Fluid samples were returned to the laboratory for processing within 2 h of collection. The sample collection set was removed from ice, the exterior of the tube cleaned and weighed on a fine balance. The collection tube containing the Weck-Cel® spear heads was removed and stored at −80°C until further analysis. Progesterone concentrations in serum were quantified using a progesterone ELISA kit (RE52231, IBL International, Germany) at baseline and on days 7, 14 and 28 to determine the phase of menstrual cycle during ring placement. Animals were necropsied on day 28 and three samples of various tissues (vagina, cervix, uterus, rectum, female genital

tract draining lymph nodes and distal lymph nodes) were taken and frozen at −80°C. Biological samples were shipped on dry ice to CEA Gif-sur-Yvette for quantification of dapivirine and darunavir by HPLC tandem mass spectrometry (HPLC-MS/MS.)

Quantification of dapivirine and darunavir in biological samples

Dapivirine and darunavir were quantified in macaque vaginal fluid, serum and tissue by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Dapivirine-d11 and darunavir-d9 (Toronto Research Chemicals, Canada) were used as internal standards. Vaginal fluid and serum sample preparation was performed as previously described.[2] Frozen tissue samples (around 50 mg) from necropsied animals were homogenized in 1 mL of ice-cold methanol/Tris 0.05 M/HCl pH 5; 70/30 (v/v) containing internal standard, using a Precellys tissue homogenizer (Bertin Technologies, France). Cellular debris was removed by spinning at 20000 **g** for 15 min at 4°C and transferring the supernatant to a fresh tube. Samples were then evaporated to dryness at 40°C with a nitrogen stream in a Turbovap LV evaporator (Biotage, UK) and re-suspended in 150 μL of initial mobile phase. The extract sample was transferred to a vial, and a 20 μL aliquot was injected into the chromatographic system. LC-MS/MS conditions were the same for the analysis of vaginal fluid, serum and tissue samples. The calibration ranges for the bioanalytical assays were 14–2000 pg/mL, 0.400–500 ng (i.e. ~20–25000 ng/g) and 1–2000 ng (i.e. ~20–25000 ng/g) for dapivirine and darunavir in serum, vaginal fluid and tissue samples, respectively. The extract samples maintained at 4°C in the autosampler were chromatographically separated using a Waters ACQUITY UPLC® System with an ACQUITY UPLC BEH RP18 shield, 2.1×100 mm, 1.7 μm column and a reversed phase gradient over a run time of 5 min. Initial conditions consisted of mobile phase A (0.4% ammonia in water) and mobile phase B (acetonitrile) at 70/30 (v/v) with a column temperature of 40°C and a flow rate of 0.400 mL/min. The gradient conditions ramped from 30% B to 100% B between 1.0 and 2.0 min, then maintained up to 2.8 min, ramped to 30% in 0.01 min and then maintained up to 5 min for re-equilibration. The MS analysis was performed on a Waters Xevo™ TQ-MS mass spectrometer operated in positive ion electrospray multiple-reaction monitoring mode (MRM) mode. Briefly, main tune parameters were as follows: capillary voltage was set up at 3 kV, source temperature was 150°C, desolvation temperature was 350°C, cone gas flow was 50 L/hour and desolvation gas flow was 650 L/h. The analytes and their corresponding internal standards were specifically monitored using a pair value cone voltage (V)/collision energy (eV) set at 55/36 and 22/12 for dapivirine and darunavir, respectively. Monitored MRM transitions were m/z 330.13/158.06, 341.16/168.08, 548.20/392.20 and 557.20/401.20 for dapivirine, dapivirine-d11, darunavir and darunavir-d9, respectively. Each transition was alternately monitored with a dwell time of 0.04 s while quadrupole resolution in both Q1 and Q3 was set at 0.7 FWHM. Under these UPLC-MS/MS conditions, dapivirine/dapivirine-d11 and darunavir/darunavir-d9 displayed mean retention times of 2.8 and 2.4 min, respectively.

Pre- and post-use content of dapivirine and darunavir rings

The initial and residual dapivirine and darunavir content of rings was quantified following manufacture and after completion of both *in vitro* release and *in vivo* pharmacokinetic testing in macaques (*n* = 4). Rings were cut into pieces of ~2 mm diameter and placed into a glass flask. Dichloromethane (95 mL) was added along with 5 mL of a 1 or 2 mg/mL solution of 19-norethindrone (depending on the initial drug loading in the ring) in methanol as internal standard. The flasks were sealed and placed in an orbital incubator (Infors HT Unitron, Switzerland; 37°C,

Table 2. Timepoints for collection of biological samples from macaques during period of ring placement

	Time after ring insertion (days)							
Sampling procedure	0	0.3	1	2	7	14	21	28
Vaginal fluid (for drug quantification)	x	x	x	x	x	x		x
Blood (for drug serum quantification)	x	x	x	x	x	x		x
Blood for progesterone quantification	x				x	x	x	x
Blood (for blood cell formula testing)	x				x	x		x
Tissue (for drug quantification)								x

60 rpm, throw 25 mm) for 72 h. One or two millilitres of the extraction mixture (again, dependent upon drug loading) was transferred to a boiling tube and evaporated to dryness. The residue was reconstituted in 10 mL of methanol with vortex mixing for 30 s and sonication for 20 min to ensure complete drug dissolution. Samples were analysed by HPLC with detection by UV absorbance at 257 nm. A 10 μL aliquot of each sample was injected onto a Phenomenex® Luna 5 μm C18(2) 100 Å column (150×4.6 mm; Phenomenex, Cheshire, UK) held at 30°C. HPLC was conducted in isocratic mode with a mobile phase of HPLC-grade water (25%) and HPLC-grade methanol (75%) at a flow rate of 0.75 mL/min. Under these conditions dapivirine, darunavir and norethindrone had mean retention times of 9.5, 3.5 and 5.1 min, respectively.

Ex vivo challenge studies on vaginal fluid samples

Vaginal fluid samples from the macaque pharmacokinetic study were extracted into 60% acetonitrile containing 0.04% ammonium hydroxide in water and shipped on dry ice to Janssen Diagnostics for *in vitro* testing of antiretroviral compound activity. The MT4-CCR5high-LTR-eGFP cell line (overexpression of CCR5 co-receptor) was kindly provided by Dr Olivia Goethals (Janssen Infectious Diseases and Vaccines). Cells were maintained in RPMI 1640 medium (Lonza) supplemented with 10% FetalClone® II serum (Sigma), 0.04% gentamicin (Gibco) and 1% Geneticin (Gibco) in a humidified incubator at 37°C, 4.5% CO_2. BaL virus (CCR5-tropic HIV-1 strain) was supplied by Advanced Biotechnologies Inc. Antiretroviral activity was determined using a cell-based HIV-1 green fluorescent protein reporter assay. A 3-fold dilution series of the extracted samples in culture medium (without Geneticin, supplemented with 2% DMSO) was made in 96-well plate format, with compound dilution determined based on the initial ring loading. MT4-CCR5high-LTR-eGFP cells (75 μL/well) and BaL virus (75 μL/well) were added to the dilution plate (50 μL/well) with a multiplicity of infection of 0.0025. By means of a liquid handler (MICROLAB® STAR; Hamilton Robotics) the 96-well assay plate was transferred to a 384-well plate (40 μL/well) for readout after 3–4 days storage. Infection of MT4-CCR5high-LTR-eGFP cells with BaL virus causes activation of the long terminal repeat promoting expression of enhanced green fluorescent protein. The fluorescence signal was measured with a fluorescence scanning microscope (Axio Vert.A1, Zeiss). The extent of inhibition of viral infection was determined as the level of fluorescence measured relative to positive controls (cells with virus, no ARVs added) correcting for basal levels observed (cells with no virus or ARVs added). Dose–response curves and IC_{50} values were calculated using GraphPad Prism.

Statistical analyses

Where appropriate, results were statistically analysed using a one-way analysis of variance, followed by *post hoc* analysis using the Tukey–Kramer multiple comparisons test. In all cases, a *P* value of <0.05 was considered significant. Analysis was conducted using GraphPad Prism.

Results and discussion

DSC data

DSC thermograms and data obtained from the various dapivirine/darunavir solid mixtures are presented in Figure 1(a) and Table 3, respectively. Dapivirine showed a primary crystalline melting transition at 220°C (Table 3, Peak 2).[11,19] The endothermic transition at 101°C in the dapivirine thermogram has been observed previously and represents the thermal transformation between dapivirine crystalline forms I and II.[11] Darunavir showed a thermal transition around 100°C, caused by desolvation (i.e. evaporation of ethanol) from the crystalline form (darunavir is an ethanolate pseudo-polymorph) and subsequent formation of the liquid drug
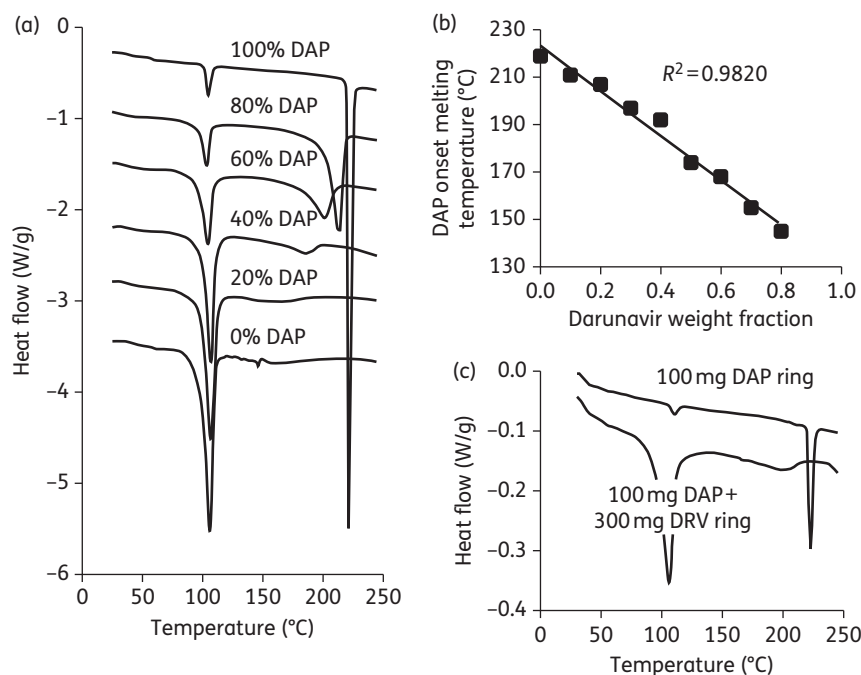
Figure 1. (a) Overlaid DSC traces of dapivirine (DAP) and darunavir (DRV) mixed in varying proportions. (b) Plot illustrating the depression of DAP onset melting temperature in the solid drug mixtures as a function of DRV concentration. (c) Overlaid DSC traces for 100 mg DAP ring and 100 mg DAP+300 mg DRV ring.

Table 3. Summary data obtained from DSC thermograms (data are means $\pm$ SD; $n \geq 4$)

Dapivirine fraction	Peak 1			Peak 2		
	onset temperature (°C)	peak temperature (°C)	enthalpy (J/g)	onset temperature (°C)	peak temperature (°C)	enthalpy (J/g)
1.00	101.27 ± 0.08	104.76 ± 0.07	9.98 ± 0.44	219.64 ± 0.07	221.78 ± 0.36	111.70 ± 1.60
0.90	99.31 ± 0.09	103.58 ± 0.08	18.99 ± 1.62	210.88 ± 1.02	218.05 ± 0.41	84.17 ± 2.92
0.80	97.81 ± 0.17	103.47 ± 0.34	25.71 ± 3.06	206.58 ± 3.05	213.16 ± 0.55	68.84 ± 9.76
0.70	97.57 ± 0.11	104.68 ± 0.25	44.88 ± 2.51	196.94 ± 0.82	208.85 ± 0.80	49.71 ± 5.56
0.60	96.69 ± 0.50	104.80 ± 0.60	53.20 ± 7.55	191.66 ± 5.66	202.93 ± 1.95	45.57 ± 3.59
0.50	95.98 ± 1.20	104.25 ± 0.47	72.47 ± 5.64	174.01 ± 2.31	194.84 ± 1.18	28.71 ± 4.96
0.40	100.12 ± 0.26	106.09 ± 0.27	79.42 ± 4.21	165.26 ± 8.12	184.84 ± 1.24	18.86 ± 2.95
0.30	96.83 ± 1.39	105.53 ± 1.02	83.86 ± 3.40	153.15 ± 4.90	174.86 ± 3.48	9.22 ± 1.73
0.20	101.47 ± 1.70	106.60 ± 0.62	92.10 ± 13.39	142.20 ± 9.48	159.20 ± 4.69	8.21 ± 2.25
0.10	99.40 ± 0.32	106.32 ± 0.48	115.70 ± 5.84	151.42 ± 6.73[a]	158.42 ± 4.10[a]	1.06 ± 0.80[a]
0.00	99.10 ± 2.96	105.60 ± 0.62	95.26 ± 10.10	—	—	—

[a]These values are less accurate due to limits in instrument sensitivity.

form.[20] At intermediate compositions, the darunavir melting temperature remained unchanged while the dapivirine melting temperature decreased and the melting endotherm peak broadened in proportion to increasing concentrations of darunavir in the mixture (Figure 1a and Table 3). The dapivirine thermal behaviour is attributed to the entropy-driven phenomenon of melting point depression (Figure 1b) associated with addition of a liquid impurity (i.e. the melted, desolvated form of darunavir). Melting point depression in two- (or multi-) component systems is directly attributed to intermolecular interactions. Often, these intermolecular interactions are hydrogen bonds, which are believed to be highly significant in melting point depression.[21] It has already been established that dapivirine forms two hydrogen bonds with Lys101 at the HIV-1 reverse transcriptase binding site and exhibits strong π-π stacking or H...π interaction with Tyr181 and Tyr188 residues. These interactions play a vital role in stabilizing the dapivirine+enzyme complex.[22] Based on these data, it is likely that the melting point depression for the dapivirine/darunavir system is attributed to hydrogen bonding, since both molecules have considerable scope for this type of intermolecular bonding.

Silicone elastomer samples containing various concentrations of either dapivirine or darunavir alone showed endothermic peaks at the normal crystalline melting temperature for each drug, and with peak areas (enthalpy values) correlating with the drug loading. By way of example, a 100 mg dapivirine ring sample is presented in Figure 1(c). A silicone elastomer sample prepared with dapivirine and darunavir concentrations equivalent to those in the 100 mg dapivirine+300 mg darunavir macaque ring [5.88% and 17.6% (w/w), respectively] showed the normal melting endotherm for darunavir. However, the normally sharp dapivirine crystalline melting transition at 220°C was not observed, being replaced by a broader melting endotherm at 150–200°C (Figure 1c), similar to that observed for the solid drug mixtures (Figure 1a).

A number of combination drug products are deliberately formulated such that the melting point of each drug in the system is depressed compared with the original drug material. Often, the final drug ratio is selected to correspond to the minimum melting temperature in the temperature–composition phase diagram, since this so called eutectic composition leads to increased solubility of each drug (and therefore improved release/dissolution). EMLA® cream (a eutectic mixture of lidocaine and prilocaine) and Nuvaring® (a combination contraceptive vaginal ring) represent two examples of marketed drug products formulated using a eutectic mixture of two different drug molecules. In the absence of other degradative drug–drug interactions, eutectic (or decreased melting temperature) systems do not pose any additional manufacturing or stability issues for pharmaceutical products.

In vitro release of dapivirine and darunavir from vaginal rings

In vitro daily and cumulative release profiles for dapivirine and darunavir from the various rings into IPA/water are presented in Figure 2. Typical of matrix-type rings, release is high on day 1 and decreases on each subsequent day (Figure 2a and b). For both drugs, *in vitro* release obeyed $t^{\frac{1}{2}}$ kinetics, as evidenced by linear cumulative release versus root time plots (Figure 2c and d; $R^2 > 0.994$, Table 4) and indicating a permeation-controlled drug release mechanism from a polymeric matrix device containing dispersed solid drug(s).[23,24] For the dapivirine-only rings, increasing the dapivirine loading from 25 to 100 mg significantly increased the dapivirine release rate from 1511 to 3464 µg/day$^{\frac{1}{2}}$ (Figure 2a and c and Table 4), in accordance with well-established mathematical models for these drug-in-polymer systems.[23,24] The additional presence of 300 mg darunavir in the combination ring further significantly enhanced the dapivirine release rate to 4201 µg/day$^{\frac{1}{2}}$ (Table 4). This enhancement may be attributed to the darunavir-induced depression of the dapivirine melting temperature (see DSC data), which would theoretically increase dapivirine solubility in the system and its subsequent release rate, and/or the formation of permeation channels in the silicone elastomer matrix resulting from release of the higher concentration darunavir component from the ring.[25] Release enhancement is also observed for darunavir when 100 mg dapivirine is incorporated
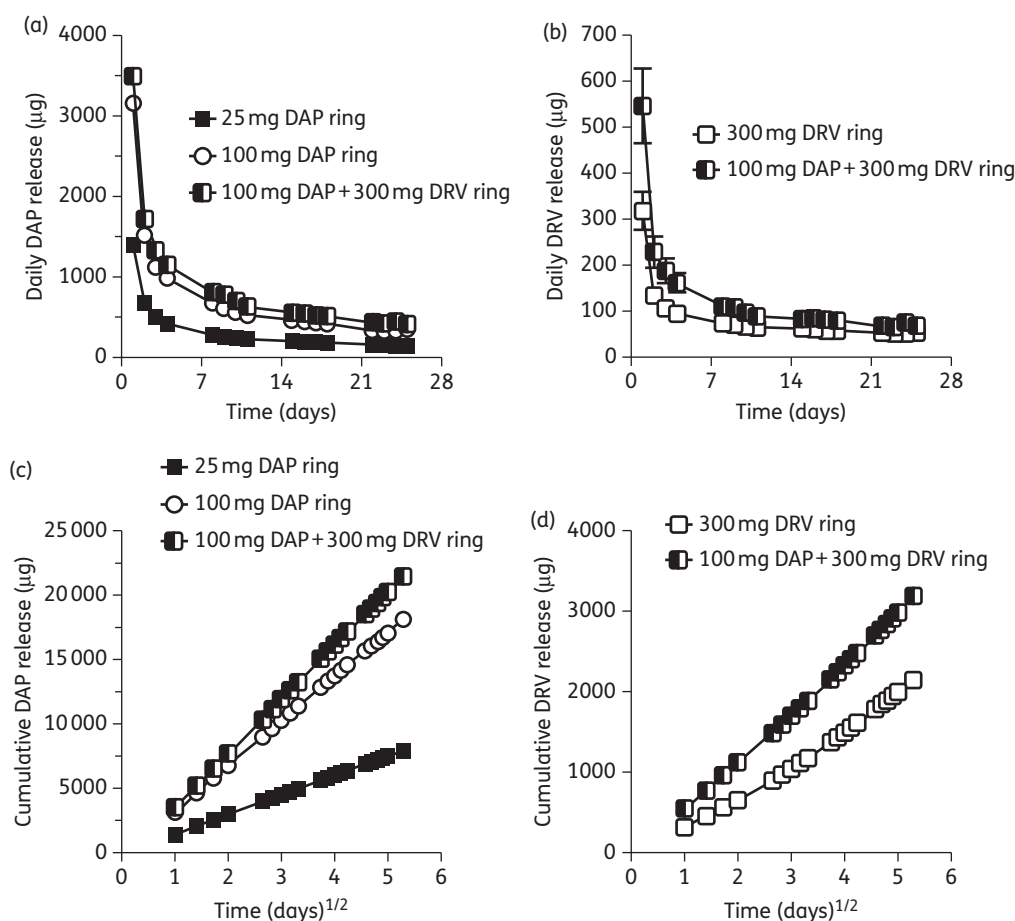
Figure 2. Daily *in vitro* release versus time (a and b) and cumulative *in vitro* release versus root time (c and d) profiles for dapivirine (DAP) and darunavir (DRV) into 100 mL of IPA/water mixture (1:1) over 28 days from a 25 mg DAP ring, a 300 mg DRV ring and a combination ring containing 100 mg DAP and 300 mg DRV. Each value is the mean of four replicates and error bars denote standard deviations.

into the ring (Figure 2b and d and Table 4). Based on the DSC data presented earlier, this darunavir enhancement cannot be attributed to reduced darunavir melting temperature (since none was observed); therefore, creation of permeation channels is assumed. In summary, for the IPA/water release experiments, the addition of 300 mg darunavir to a 100 mg dapivirine ring results in a 21.2% increase in the dapivirine release rate (Figure 2c and Table 4), while the addition of 100 mg dapivirine to a 300 mg darunavir ring produces a 42.5% increase in the darunavir release rate (Figure 2d and Table 4). *In vitro* release of dapivirine and darunavir into SVF (Figure 3) highlights the extent to which drug solubility in the surrounding fluid controls release from a ring; both drugs are poorly water soluble, such that release into SVF is significantly less than that into IPA/water. However, it is interesting to note that dapivirine release into SVF is significantly increased upon increasing the drug loading from 25 mg to 100 mg (irrespective of whether darunavir is present or not). Based on dapivirine aqueous solubility values ($\sim$2.5 μg/mL in pH 4 buffer and $>$1 μg/mL in water; R. K. Malcolm, unpublished data), the mean day 1 release of dapivirine (179 μg) from the 100 mg ring is likely very close to the solubility limit of dapivirine in SVF. In fact, for all dapivirine rings released into SVF, sink conditions (under which drug concentrations are not permitted to exceed 10% of saturation solubility) are probably not in operation.

Pharmacokinetic studies in cynomolgus macaques

Three different ring formulations were tested for pharmacokinetics in macaques: 25 mg dapivirine-only ring, 100 mg dapivirine + 300 mg darunavir ring, and 300 mg darunavir-only ring. The concentration versus time plots for dapivirine and darunavir in serum and vaginal fluid during the 28 day period of ring placement are presented in Figure 4. Pharmacokinetic parameters for dapivirine and darunavir in serum and vaginal fluid are summarized in Table 5. Initial serum concentrations for both ARVs were $\sim$10^2 pg/mL, similar to plasma concentrations previously measured in women using dapivirine rings.[2] Dapivirine serum concentrations were maintained at these levels throughout the entire period of ring use (Figure 4a), while darunavir concentrations decreased slightly over time (Figure 4b). Dapivirine serum concentrations were similar for the 25 mg dapivirine-only ring and the 100 mg dapivirine + 300 mg darunavir ring; darunavir serum concentrations were consistently higher for the 100 mg dapivirine + 300 mg darunavir ring compared with the 300 mg darunavir-only ring, although the differences were not always statistically significant. Dapivirine concentrations in vaginal fluid were maintained within the range 2×10^3 – 2×10^4 ng/g over the study period, with concentrations consistently (but not always significantly) higher for the 100 mg dapivirine-loaded combination ring (Figure 4c). Human

Table 4. Summary *in vitro* release data for dapivirine (DAP) and darunavir (DRV) for the various silicone elastomer ring formulations into 1:1 IPA/water mixture and SVF release media

| Ring formulation	Drug	Release medium	Release rate (μg/day$^{\frac{1}{2}}$)	R^2 value
25 mg DAP	DAP	IPA/water	1511	0.9998
100 mg DAP	DAP	IPA/water	3464	0.9998
100 mg DAP+300 mg DRV	DAP	IPA/water	4201	0.9999
300 mg DRV	DRV	IPA/water	430.4	0.9942
100 mg DAP+300 mg DRV	DRV	IPA/water	613.4	0.9988
25 mg DAP	DAP	SVF	196.8	0.9702
100 mg DAP	DAP	SVF	365.6	0.9980
100 mg DAP+300 mg DRV	DAP	SVF	361.0	0.9979
300 mg DRV	DRV	SVF	197.4	0.9988
100 mg DAP+300 mg DRV	DRV	SVF	181.1	0.9968

Release rates are the gradients obtained from linear regression analysis of the mean cumulative release versus root time plots (Figures 1c, d, 2c and d). R^2 values, representing the Pearson coefficient of determination, are also reported for the root time plots; values close to unity demonstrate matrix-type root-time release kinetics.
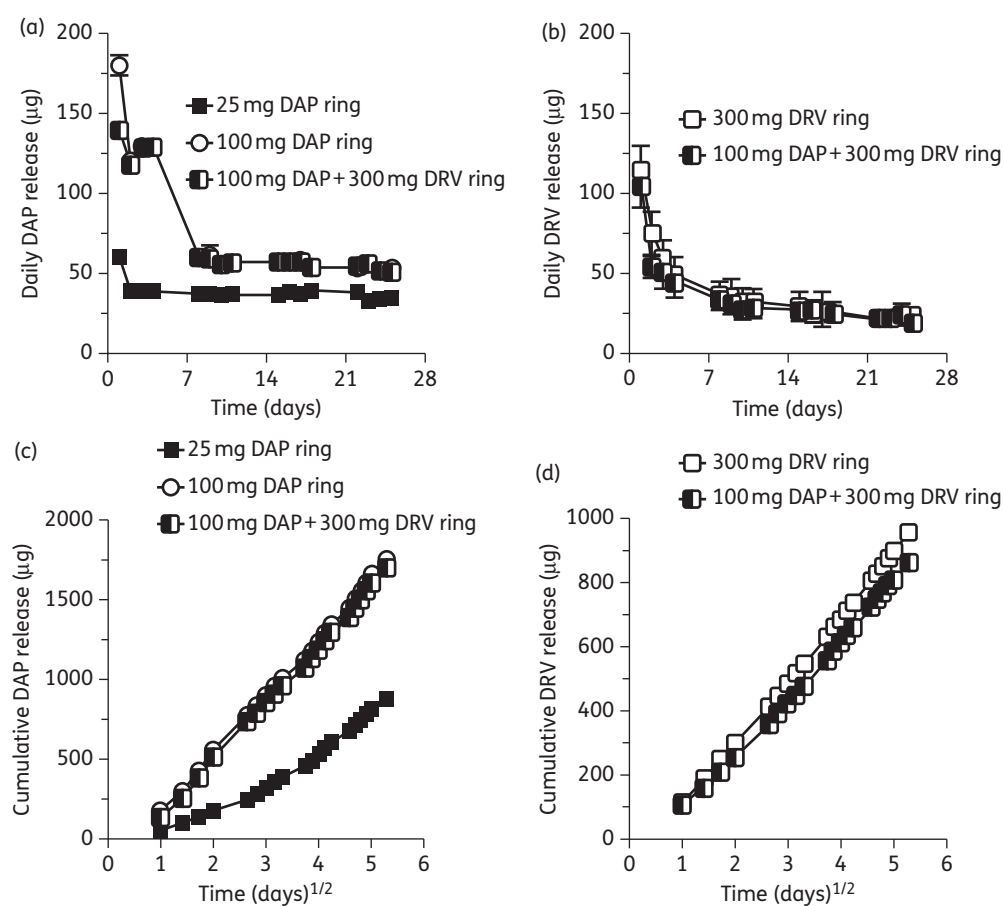
Figure 3. Daily *in vitro* release versus time (a and b) and cumulative *in vitro* release versus root time (c and d) profiles for dapivirine (DAP) and darunavir (DRV) into 100 mL of SVF over 28 days from a 25 mg DAP ring, a 100 mg DAP ring, a 300 mg DRV ring and a combination ring containing 100 mg DAP and 300 mg DRV. Each value is the mean of four replicates and error bars denote standard deviations.

vaginal fluid concentrations for 25 mg dapivirine matrix and 25 mg dapivirine reservoir-type rings have previously been reported as ranging from 4×10^3 to 3×10^6 ng/g.[2] These higher

dapivirine concentrations are likely attributed to use of a different type of silicone elastomer system and the larger size (and therefore greater surface area for drug release) of the human-sized
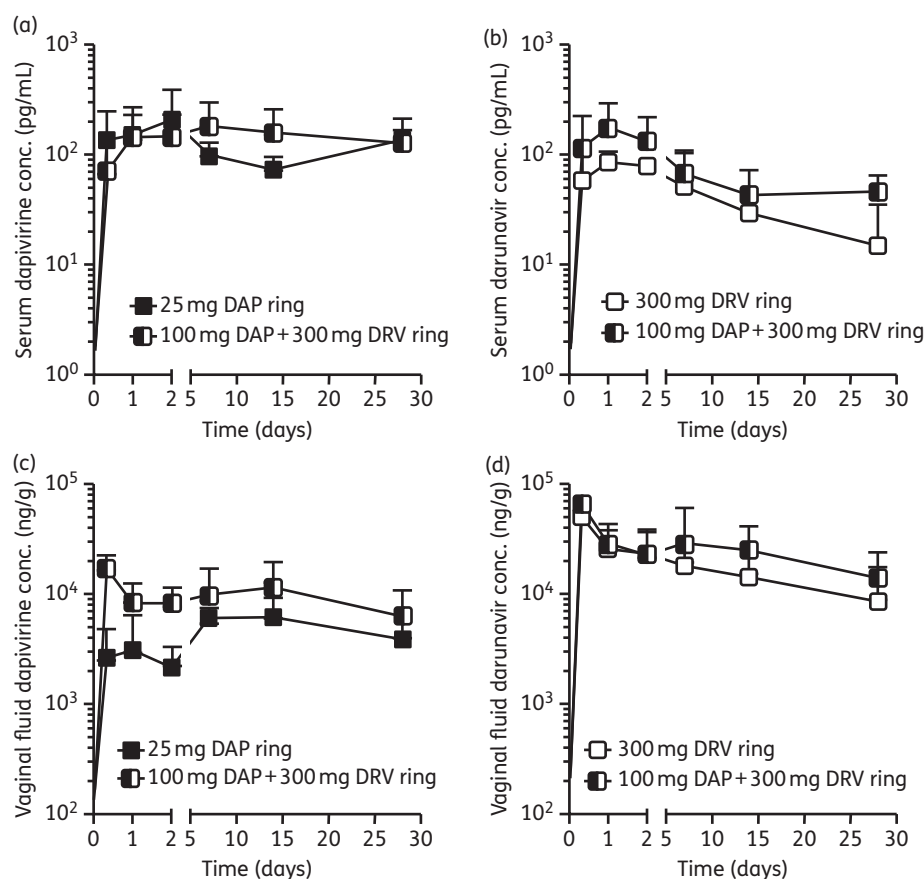
Figure 4. Serum (a and b) and vaginal fluid (c and d) concentration versus time profiles for dapivirine (DAP) and darunavir (DRV) during 28 day placement in cynomolgus macaques of silicone elastomer matrix-type rings containing 25 mg DAP, 300 mg DRV and a combination of 100 mg DAP and 300 mg DRV. Each value is the mean of four replicates and error bars denote standard deviations.

Table 5. Statistical pharmacokinetic parameters for dapivirine (DAP) and darunavir (DRV) in macaque serum and vaginal fluid following placement of vaginal rings containing 25 mg DAP, 300 mg DRV and 100 mg DAP+300 mg DRV

| Drug	Ring formulation	Vaginal fluid			Serum		
		C_{max} (ng/g)	T_{max}^a (h)	AUC (µg·h/g)	C_{max} (pg/mL)	T_{max}^a (h)	AUC (pg·h/mL)
DAP	25 mg DAP	7591 ± 2508	168	3336 ± 854	250 ± 150	48	74851 ± 35024
DAP	100 mg DAP+300 mg DRV	19260 ± 5675	8	6336 ± 3999	206 ± 108	108[b]	100945 ± 51871
DRV	300 mg DRV	50650 ± 12425	8	10508 ± 1996	101 ± 33	24	27612 ± 12201
DRV	100 mg DAP+300 mg DRV	69916 ± 11798	8	15863 ± 11401	178 ± 116	24	43009 ± 16236

[a]The most common point reporting the maximum concentration.
[b]No timepoint was most common; value reported is the mean of the two middle timepoints.

rings (56 mm outer diameter, 7.6 mm cross-sectional diameter). However, they might also be attributed to the different pH environments in human (pH 4.5) and macaque (pH 7) vaginas; dapivirine is a weak base (pK$_a$=5.54 at 25°C[26]) and therefore is present mostly in its protonated form at human vaginal pH, whereas the free base form predominates at macaque vaginal pH. Darunavir concentrations tended to decrease with time (Figure 4d), ranging from 7×10^4 ng/g at 8 h after ring placement to 9×10^3 ng/g on day 28. Maximum or near-maximum vaginal

fluid and serum concentrations for dapivirine and darunavir were mostly observed at or close to the initial 8 h sampling time-point, indicating relatively rapid release of the drugs from the rings and subsequent absorption.

The range of dapivirine and darunavir concentrations measured in the various macaque tissues following necropsy on day 28 decreased according to the following rank order: vagina (1.8×10^3 – 3.8×10^3 ng/g) > cervix (9.4×10^1 – 3.9×10^2 ng/g) > uterus (0–108 ng/g) > rectum (0–40 ng/g) > axillary lymph
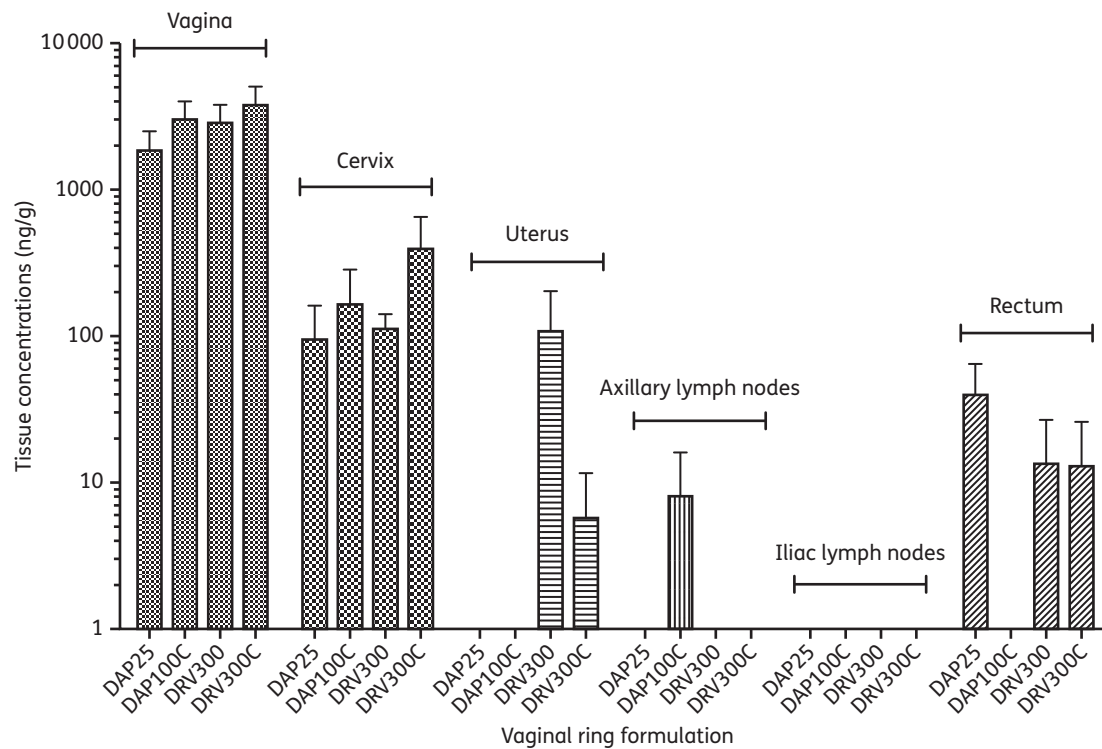
Figure 5. Dapivirine and darunavir concentrations in various macaque tissues collected at necropsy and following 28 day placement of silicone elastomer matrix-type rings containing either 25 mg dapivirine (DAP25) or 300 mg darunavir (DRV300) or a combination of 100 mg dapivirine and 300 mg darunavir (DAP100C and DRV300C).

nodes (0–8 ng/g) >iliac lymph node (no drug levels detected; Figure 5). The greater drug concentrations measured in the vagina relative to the cervix were consistent with the observation following necropsy that rings were located in the mid-vagina; by comparison, additional studies with smaller rings (20×4.5 mm) are ongoing and clearly show the smaller ring located close to the cervix. The relatively high drug concentrations in both the vagina and cervix are important, since these tissues are implicated as the primary infection sites for sexual transmission of HIV-1. In fact, the highest concentration of HIV-infectable cells (i.e. CD4+ T lymphocytes, macrophages and Langerhans cells) in the female genital tract are found in the cervical tissue, with significantly lower numbers found in the vaginal tissue.[27] Approximately 2-fold higher dapivirine concentrations have previously been measured in vaginal tissue biopsies sampled adjacent to the ring location compared with ectocervical tissue biopsies for two different reservoir-type rings in humans.[3] The detection of dapivirine and darunavir in non-vaginal tissues is attributed to passive diffusion, although darunavir concentrations in the uterus (dapivirine was not measured in the uterus) may also have resulted from a first uterine pass effect.[28,29] We have recently reported both vaginal-to-rectal and rectal-to-vaginal transfer of CMPD167, an experimental entry inhibitor antiretroviral drug, following vaginal administration of gels and rings and rectal gel administration.[30] Dapivirine and darunavir tissue concentrations did not vary significantly across the different ring formulations, although some general trends (and exceptions) can be observed. For example, dapivirine concentrations in vaginal and cervical tissues (Figure 5) were generally 2-fold greater for the combination ring (containing 100 mg dapivirine) compared with the

25 mg dapivirine-only ring, perfectly reflecting the prediction based on the Higuchi equation that a 4-fold increase in drug loading is required to double the drug release rate in a permeation-controlled matrix-type drug delivery system.[23,31] Also, concentrations of darunavir in the vaginal and cervical tissues were generally higher for the combination ring (containing 300 mg darunavir) compared with the 300 mg darunavir-only ring, mimicking the *in vitro* release data into IPA/water (Figure 2d) and suggesting that incorporation of dapivirine increases the darunavir release rate.

Endogenous serum progesterone concentrations were also measured at various timepoints (Table 2) during ring placement to determine the stage of menstrual cycle for each macaque (data are presented in the Supplementary information). Generally, the follicular and luteal phases were readily identified by progesterone serum levels of <1 and 2–15 ng/mL, respectively (Figure S1 and Figure S2, both available as Supplementary data at *JAC* Online). There was no obvious correlation between the stage of menstrual cycle and drug concentrations in vaginal fluid (Figure S1 and Figure S2) or serum (data not shown). Exogenous progestin administration, in the form of subcutaneously administered medroxyprogesterone acetate (Depo-Provera), has previously been shown to significantly influence the pharmacokinetics of antiretroviral-releasing vaginal rings in macaques.[9]

Ex vivo activity of dapivirine and darunavir against HIV challenge

IC$_{50}$ values calculated against HIV-1 BaL from samples taken at each timepoint are displayed in Figure 6 for all of the rings.

Mean IC_{50} values for dapivirine from the 25 mg dapivirine ring ranged from 0.09 to 0.14 ng/mL (two samples excluded because of ambiguous values). On average, dapivirine concentrations in vaginal fluid were >15 000-fold greater than the calculated IC_{50} value (ng/mL) during the 28 day study period. However, because of the relatively small pre-dilution step (1:3), solvent effects on the dose–response curve cannot be excluded and hence the calculated IC_{50} value should be viewed with caution. Several of the samples from the 300 mg darunavir ring study provided ambiguous IC_{50} values or failed to provide an appropriate dose–response curve. Mean IC_{50} values calculated on samples taken at 8 h and 7 days ranged from 1.36 to 1.86 ng/mL. Over this time period the darunavir concentration in vaginal fluid was >17 000-fold the calculated IC_{50} value. However, the data from samples taken outside this time frame did not allow for robust IC_{50} value determination. Mean IC_{50} values calculated for the combination ring ranged from 0.28 to 1.31 ng/mL. On average, the dapivirine and darunavir concentrations in vaginal fluid (ng/g) were >20 000-fold the determined IC_{50} values over the 28 day study period. Note that the IC_{50} calculated for the combination dapivirine+darunavir ring reflects the combined compound concentration of dapivirine and darunavir within the extracted vaginal fluid sample.

In general, the data support the activity of both drugs when present in vaginal fluid at sufficient concentration. However, the methodology required to effectively extract and process these highly hydrophobic drugs, and dapivirine in particular, reduces the accuracy of these measurements owing to the presence of residual organic solvent in the test sample. Further work is required to develop a method that would allow for compound testing in the *in situ* vaginal fluid, particularly for highly hydrophobic compounds with relatively poor aqueous solubility like many experimental NNRTIs.

Residual dapivirine and darunavir in vaginal rings

The quantities of dapivirine and darunavir released from the rings during *in vitro* release testing and pharmacokinetic testing in macaques were calculated according to the equation $Q=L-R$, where Q is the quantity of drug released, L is the initial drug loading and R is the residual drug content. L was determined for a sample ($n=4$) of rings immediately after manufacture using the previously described solvent extraction method, and the value was assumed to apply to all other rings within the same batch. R was also determined using the solvent extraction method for rings ($n=4$ per set) following completion of *in vitro* release and pharmacokinetic testing. Given that L values could not be determined for the rings that underwent testing (due to the destructive nature of the content determination method), the Q values reported in Table 6 are considered approximate. For both antiretroviral drugs, *in vitro* release into IPA/water was significantly greater than into SVF, reflecting the poor aqueous solubility of dapivirine and darunavir. In the macaque pharmacokinetic studies, the quantity of dapivirine administered was greater for the
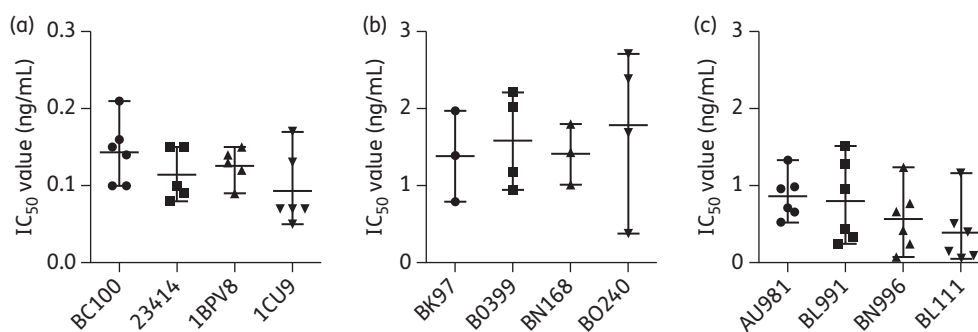
Figure 6. IC_{50} (HIV-1 BaL) values determined from macaque vaginal fluid samples for (a) 25 mg dapivirine ring, (b) 300 mg darunavir ring and (c) 100 mg dapivirine+300 mg darunavir ring. *x*-axis labels refer to individual macaque code.

Table 6. Quantity of dapivirine (DAP) and darunavir (DRV) released from vaginal rings during *in vitro* release testing and *in vivo* pharmacokinetic (PK) studies in macaques

Ring formulation	Dapivirine			Darunavir		
	in vitro release testing			*in vitro* release testing		
	IPA/water	SVF	PK study	IPA/water	SVF	PK study
25 mg DAP	7.89±0.04	0.88±0.01	0.66±0.39	—	—	—
100 mg DAP+300 mg DRV	21.48±0.07	1.70±0.01	12.62±1.68	3.19±0.16	0.86±0.13	27.41±4.82
300 mg DRV	—	—	—	2.14±0.06	0.96±0.19	36.04±6.56

In vitro release testing values are presented as the mean cumulative release (±SD) of rings ($n=4$) into IPA/water and SVF. PK study values represent mean amounts of drug released ($n=4$; ±SD). These values were calculated from residual content values determined using solvent extraction subtracted from a mean batch content determination conducted on rings manufactured in the same batch. The standard deviation is that of the four determined residual content values and does not consider the variation in initial content of the manufactured rings. All values are quoted in mg.

higher-loaded combination ring (12.62 ± 1.68 mg) compared with the 25 mg dapivirine-only ring (0.66 ± 0.39 mg), while the quantity of darunavir administered was not significantly different between the 300 mg darunavir and 100 mg dapivirine + 300 mg darunavir rings.

Given that a 25 mg dapivirine-only silicone elastomer vaginal ring is currently being tested in Phase III human clinical studies, and based on the supposition that combination antiretroviral microbicides targeting different stages of the HIV-1 replication cycle are likely to provide increased breadth and degree of protection against vaginal HIV transmission compared with single active molecules, we conclude that the results of this study strongly support progress of a combination dapivirine + darunavir silicone elastomer vaginal ring into early stage clinical studies. The potential of protease inhibitors as vaginal microbicides, either alone or in combination, also warrants further investigation.

Acknowledgements

Funding

Transparency declarations

O. L., L. V., M. F. and J. V. R. are employees of Janssen Diagnostics (a Johnson & Johnson Company). O. L. and J. V. R. own stocks in Johnson & Johnson. The remaining authors have none to declare.

Supplementary data

Figure S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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